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Development of PNA-based systems for food safety and molecular biology

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"*Science is nothing else than the search to discover unity in the wild variety of nature, or more exactly, in the variety of our experience.*"

(J. Bronowski; Science and human values)

"*The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...' *"

Isaac Asimov

"*The opposite of a right statement is a wrong statement. The opposite of a deep truth can also be a deep truth.*"

Niels Bohr

A Federica

A Gabriella e Giuseppe

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Contents

Chapter 1

Introduction

1. 1 FOOD SAFETY AND QUALITY: TWO IMPORTANT AND CHALLENGING PUBLIC ISSUES

Food safety and quality are progressively gaining importance as matters of public concern and debate. Consumers demand rules and systems for tracing the origin and the composition of foods, in order to be reassured about the safety and authenticity of what they eat. On the other hand, istitutional and transnational bodies are working to strengthen food related policies and to create new legal frameworks in order to assure high standard of food safety and quality.

The food and drink industry is a leading industrial sector in many countries; in the European Union (EU), this industry has an annual production which is worthed almost 600 billion €, about 15% of the total manufacturing output.

On the basis of international comparisons, the EU results the world's largest producer of food and drink products with over 2.6 million employees, of which 30% are in small and medium enterprises that make the food and drink industry the third-largest industrial employer of the EU. Moreover, the agricultural sector has an annual production of about 220 billion € and provides the equivalent of 7.5 million full-time jobs (Commission of the European Communities, 2000).

Since the food production chain is becoming increasingly complex, new approaches for food safety, quality and traceability are required both at the scientific and legislative level in order to ensure public health and contrast fraudulent or deceptive practices.

An increasing number of food safety problems and food related frauds is raising the consumers' concerns. In industrialized countries, the percentage of the population suffering from foodborne diseases each year has been reported to be up to 30% (WHO website). In the United States of America (USA), for example, it has been estimated that around 76 million cases are related to foodborne diseases, resulting in 300,000 hospitalizations and 5,000 deaths each year (CDC website). Although it is not possible to define the global incidence of foodborne disease, it has been reported that in 2005 alone, 1.8 million people died from diarrhoeal diseases; a high percentage of these cases can be attributed to contamination of food and drinking water.

The EU has a comprehensive food safety strategy based on three core elements: legislation on the safety of food and animal feed, sound scientific advice on which to base decisions and enforcement and control. The EU strategic priorities related to food safety has been stated in the 'White Paper on Food Safety' of January 12, 2000: to create a European Food Safety Authority (EFSA), which was set up in January 2002 and is now the european reference body

to advise for risk assessment on food and feed safety, animal health and welfare, nutrition, plant protection and plant health; to enforce a "farm to table approach" in food legislation; to establish the principle that feed and food operators have primary responsibility for food safety and implement it through surveillance and control strategies.

Beside the efforts to update policies and legal frameworks, diagnostics play a pivotal role in assuring food safety, quality and authenticity. A wide range of techniques has been developed in the last decades since the potential food related threats are numerous and heterogeneous.

The main reasons to develop effective diagnostic methods for the food industry are:

i) the presence of foodborne diseases from microorganisms. Salmonellosis is still a major problem in most countries. It is caused by the Salmonella bacteria and associated to a wide variety of foods (raw meats, poultry, eggs, milk and dairy products, fishes, shrimp,s frog legs, yeast, coconuts, sauces and salad dressings, cake mixes, cream-filled desserts and toppings, dried gelatin, peanut butter, cocoa, and chocolate). It gives rise to diseases involving fever, headache, nausea, vomiting, abdominal pain and diarrhoea. It has bees estimated that from 2 to 4 million cases of salmonellosis occur only in the U.S. annually. Enterohaemorrhagic (causing intestinal bleeding) *E. coli*, e.g. *E.coli* O157, and listeriosis (caused by *Listeria monocytogenes*) are other important foodborne diseases. Their incidence is relatively low but they can be cause severe and sometimes fatal health consequences especially among elderly, infant and children and immunocompromised patients.

Campylobacteriosis has been reported to be a widespread infection and in some countries the reported cases surpass the incidence of salmonellosis. Caused by certain species of Campylobacter bacteria the foodborne cases are related to foods such as raw milk, raw or undercooked poultry and drinking water. Severe abdominal pain, fever, nausea and diarrhoea are among the acute health effects of campylobacteriosis, but in two to ten per cent of cases the infection is related to chronic health problems, including reactive arthritis and neurological disorders.

Recently, food-borne and water-borne viruses such as Noroviruses are gaining importance: this viruses seem to account for the majority (around 90%) of the non-bacterial gastroenteritis outbreacks worldwide.

ii) the presence of naturally occuring toxic compounds such as mycotoxins, marine biotoxins, cyanogenic glycosides and toxins occurring in poisonous mushrooms;

iii) the presence of potentially dangerous chemical compounds generated during food processing such as acrylamide;

iv) Persistant Organic Pollutants (POPs) that accumulate in the environment and the human body such as dioxins and polychlorinated biphenyls (PCBs), as byproducts of some industrial processes and waste incineration may contaminate food through pollution of air, water and soil;

v) the presence of potentially dangerous metals such as lead, mercury and cadmium in foods;

vi) the presence of unconventional agents as in the case of bovine spongiform encephalopathy (BSE, or "mad cow disease") that is associated with a variant of the Creutzfeldt-Jakob Disease (vCJD) in humans;

vii) intentional addition of dangerous substances to simulate a color or to mask a color;

iix) undeclared potential presence of foods such as in the case of genetically modified organisms (GMOs) which must be declared on the label. In some cases like allergenic foods, even the potential presence of food traces have to be declared;

ix) alteration of food authenticity by substitution, in part or whole, of cheaper and inferior food products for high-cost foods. This types of food often must be "conform to the description provided by the producer or processor", in which the process history of a product or ingredient, its geographic region of origin, or the species or variety of the ingredient are precisely defined. Examples are the foods defined in the EU under the categories of *Protected Designation of Origin* (PDO), *Protected Geographical Indication* (PGI) and *Traditional Specialty Guaranteed* (TSG) like Allgäuer Emmentaler cheese (Germany), Prosciutto di Parma ham (Italy), Parmigiano-Reggiano cheese (Italy), Camembert de Normandie (France) and Feta cheese (Greece), Styrian pumpkin seed oil (Austria) and Newcastle brown ale (United Kingdom).

The increasing complexity related to the food industry requires the needs to constantly improve the diagnostic methods in order to assure the food safety and quality and help to contrast frauds. A wide spectrum of methods ranging from analytical chemistry to molecular biology have been adapted or newly developed for for foods analysis (Table 1.1). The techniques most employed for food analysis include: i) chromatographic techniques such as *Gas Chromatography* (GC), *High-Performance Liquid Chromatography* (HPLC) and *Thin-Layer Chromatography* (TLC); ii) spectroscopic techniques such as *UV-Vis spectrophotometry, Infrared (IR) spectroscopy, Nuclear Magnetic Resonance (NMR) spectroscopy;* iii) stable isotope analysis such as *Isotope ratio mass spectrometry (IR-MS)* or *Site-specific Natural Isotope Fractionation Nuclear Magnetic Resonance spectroscopy (SNIF-NMR)*; iv) enzyme Immunoassay (*EIA*) such as *Enzyme*-L*inked ImmunoSorbent Assays* (*ELISA*) and v) DNA- and RNA-based methods such as the Polymerase Chain Reaction

(PCR) based methods, isothermal based amplification methods like Nucleic Acid-Sequence Based Amplification (NASBA) or microarrays.

All these methods or combination of them are currently playing an important role in food analysis.

However, the needs for more efficient, cheep, esay and fast methods is growing fast and scientists are often requested to improve existing methods in order to face new applications. Moreover, emerging foodborne pathogens, such as Noroviruses, pose constantly new challenges from both legislative and scientific points of view.

Table 1.1. Example of methods suitable for analysis in food safety, quality and authenticity

Test method	Commodity	Characteristics to be revealed	Reference
PCR	Tomato, maize, soybean	Transgenecity	Popping B, 2002
Multiplex PCR	feed and foodstuffs	OGM detection	Germini A et al., 2004a
Qualitative PCR	Meat	Presence or absence of pork	Popping B, 2002
Quantitative real-time PCR	Meat	Meat quantification	Lopez-Andreo M et al ., 2006
AFLP	Salmon	Distinguishing 10 different salmon-like species	Russell VJ et al ., 2000
	Fish and seafood	Identification of the species of origin	Maldini M et al ., 2006
DNA test (protein-based BSE test	Cattle traceability	Geographical origin/ BSE tested	Popping B, 2002
HPLC	Cow, ewe, goat, buffalo	Identification of the species of origin	De La Fuente MA and Juarez M, 2005
	Chili powder	Classification of chili powders	Forgacs E and Cserhati T, 2006
ELISA	Cow, ewe, goat, buffalo	Identification of the species of origin	De La Fuente and Juarez, 2005
LC-ESI-MS/MS	gluten-free food products	detection of free and bound fumonisins	Dall'Asta C et al., 2009
Microarray	flour samples	OGM detection (soybean and maize)	Germini A et al.,2005
NASBA	Ready-to-eat foods; water	Norovirus detection	Jean J et al., 2006; Rutjes SA et al., 2006

1.2 NOROVIRUS

1.2.1 Introduction

Norovirus (NoV) (Figure 1.1) is one of the four viruses belonging to the *Caliciviridae* family along with Sapovirus (the other calicivirus affecting human) and other two members that infect animals Lago- and Vesivirus (Table 1.2); this visus family consist of small (27-35 nm) non enveloped viruses with an icosahedral structure and a positive-sense, single-stranded RNA (ssRNA) genome of approximately 7.5 kb.

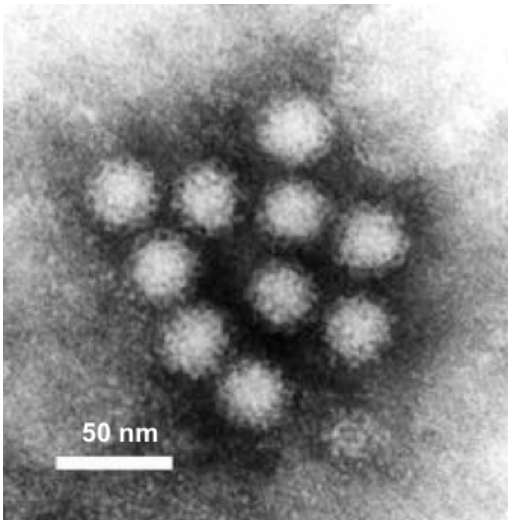


Figure 1.1. Human norovirus picture acquired using the negative-stain transmission electron microscopy technique (F.P. Williams, U.S. Environmental Protection Agency)

Norovirus is considered the most common cause of non-bacterial intestinal desease world-wide, accounting for greater than 90% of viral gastroenteritis and around half of all outbreaks worldwide (Patel MM et al., 2009).

Table 1.2. Genus and species of the calicivirus family

Genus	Species
Vesivirus	Vescicular exanthema of swine virus (VESV) Feline Calicivirus (FCV)
Lagovirus	Rabbit hemorrhagic disease virus (RHDV) European brown hare syndrome virus (EBHSV)
Norovirus	Norwalk Virus (NV)
Sapovirus	Sapporo virus (SV)

Even though the first description of the "winter vomiting disease" was reported in 1929 and evidences for the association of that disease to a virus were reported during the 1940s and 1950s, it was only in the 1972 that Norovirus was discovered as etiological agent of this syndrome examining stool samples by immunoelectron microscopy (IEM) (Kapikian AZ et al., 1972). Since the virus was first identified in samples coming from an outbreak in Norwalk, Ohio, the virus was named Norwalk virus. The virus genus, previously denoted as Norwalk-like viruses and after as "small round structured" viruses, was finally defined Norovirus by the International Committee on Taxonomy of Virus (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/00.012.0.03.htm) in 2002.

Dramatic improvements in the understanding of the molecular details of Norovirus were achieved in 1990 with the molecular cloning and characterization of the virus genome (Xi JN et al., 1990), thus unlocking the possibility for further studies on the epidemiology of Norovirus and for the development of a wide spectrum of molecular diagnostic assays.

1.2.2 Classification

Since an effective cell culture system has not yet been settled up, one of the current proposed Norovirus classification relies on sequence similarity evaluations of the capsid protein sequence (Zheng DP et al., 2006); however, no official standard classification rules have been enstablished at the international level. Currently, five genogrups (G) have been defined: the human norovirus (HuNoV) involves genogroups GI, GII and GIV (Fankhauser RL et al., 2002), the cattle specific strains are comprised in GIII (Ando T et al., 2000), whereas the murin norovirus in the GIV (Hsu CC et al., 2007). According to the criteria proposed by Zheng and colleagues, the range in terms of amino acid differences for the same genogroup is 44.9-61.4%. Following this classification, the five norovirus genogroups are further divided into 29 genetic clusters characterised by a pairwise distance range of 14.3-43.8%. The genotype clustering process has led to the definition of 31 or 32 clusters, according to other works (Wang QH et al., 2007; Patel MM et al., 2009).

1.2.3 Virus structure and genomic organization

The NoVs are non-enveloped viruses with a spherical shaped capsid which exhibit a T = 3 icosahedral symmetry, composed of 180 units of the capsid protein organized into 90 dimers. The subunits are composed of two distinct domains named S, involved in the formation of the shell and P, which forms a region protruding from the shell (Prasad BV et al., 1999). This

architectural framework seems to be shared among the four *Caliciviridae* genera even if important structural variations have been reported (Chen R et al., 2004).

The NoVs genome is an approximately 7.5 kb long, positive-sense single stranded RNA organized into 3 Open Reading Frames (ORFs) (Figure 1.2).

Figure 1.2. Norovirus genome organization

The ORF1 encodes a single polyprotein processed by a viral 3C-like protease to provide non structural proteins: p48 which seems to interfere with the cell transport system; NTPase, the nucleoside triphosphatase; p22, a protein with unidentified function, localized along the NoV genome in a position similar to the position of the 3A protein in picornavirus genomes; VPg which has been found covalently linked to genomic and subgenomic mRNAs in the animal caliciviruses, the viral protease $3CL^{pro}$ and the RNA-dependent RNA polymerase, RdRp.

ORF2 encodes the major capsid protein (VP1) which provides the functionalities for capsid assembly, receptor recognition, host specificity, strain diversity and immunogenicity (Chen R et al., 2004) and ORF3 encodes a minor structural protein (VP2) involved in virus particle stability (Hardy ME, 2005).

The NoV genome region including the C-terminal portion of the gene coding the RNA-dependent RNA polymerase and the N-terminal portion of the gene coding the VP1 protein was demonstrated to be the most conserved region in the NoV genome (Katayama K et al., 2002). This genome sequence, defined "ORF1-ORF2 junction region" was extensively targeted in molecular assays based on nucleic acid amplification (Kageyama et al., 2003; Höhne M and Schreier E, 2004; Jothikumar N et al., 2005; Mohamed N et al., 2006; Ishida S et al., 2008).

1.2.4 Clinical manifestations, immunology and pathogenesis

NoV infection is usually characterized by acute onset of nausea, vomiting, myalgias, abdominal cramps, low-grade fever and diarrhea with an incubation time of 24-48 h; however the syntoms may vary both in terms of manifestation and duration: vomiting seems to be prevalent in persons above 1 year of age, whereas diarrhea is prevalent in persons under 1

year of age (Patel MM et al., 2009). The symptoms usually last 2 - 3 days, but exceptions in some specific cases have been reported (Rockx B et al., 2002; Lopman BA et al., 2004). Even if NoV illness resolves in few days, recently developed sensitive molecular diagnostic methods have revealed the persistence of NoV in patients' stool up to three weeks after infection (Rockx B et al., 2002). The prolonged viral presence in stool poses an imporant issue for the public health management so that a correct comprehension of this mechanism might be helpful in outbreak control and prevention.

NoVs infection affects persons of all ages and is generally self-limiting; electrolyte replacement and sugar supplementation may be administered as oral solution to facilitate the rehydratation. However, in some peculiar settings or among particularly exposed persons, the mildness of the NoV illness can be compromised and the respective healing course may be more difficult and with other clinical complications; deaths have been reported for elderly patients (Harrys JP et al., 2008) and described in nursing homes.

Some evidences of association between NoV and necrotizing enterocolitis have been reported (Turcios-Ruiz RM et al., 2008). Chronic NoV infections have been observed in immunocompromised patients (Kaufman SS et al., 2005; Siebenga JJ et al., 2008; Westhoff TH et al., 2009) and unusual clinical manifestations, such as disseminated intravascular coagulation occurred during an outbreak among British soldiers in Afghanistan (Brown D et al., 2002) and a possibly NoV-associated encephalopathy in a 23-month patient, who showed the viral genome was detected in the stool, serum and cerebrospinal fluid (CSF) detected by reverse transcription polymerase chain reaction (Ito S et al., 2006), have also been reported.

Although virus-host interactions, in terms of localization and molecular interactions, have not yet been completely defined, a study demonstrated that the binding of recombinant NoV-like particles (rNV VLPs) maily occours at the villi level in the gastroduodenal junction (Marionneau S et al., 2002). Furthermore, NoVs VPLs have been revealed to bind to the ABH human histo-blood group antigens (HBGAs), a family of complex glycans expressed in the gut and on respiratory ephitelia and found on the surface of red blood cells. The NoV ability to bind several HBGAs has been demonstrated using VLPs from the GII.4 genocluster and it has been suggested as mechanism strictly related to the antigenic variations, that allow NoV to escape from herd immunity. The evolution within specific regions in the capsid seems to lead this process (Donaldson EF et al., 2008).

Human volunteer studies have revealed that a short-lived immunity is developed during NoV infection; however the immunity may be strain or genogroup specific and disappear after 2 - 3 years since the subjects challenged with the same NoV inoculum developed the illness again.

Long-term immunity response has not been clearly understood and challenge studies attempting to investigate this point showed divergent results: challenge studies have shown the possibility to re-infect individuals with the same virus strain after 27-42 months after the first virus challenge. Other studies, however, have shown that genetically susceptible individuals have never developed illness upon challenge, suggesting a sort of long-term acquired immunity (Donaldson EF et al., 2008).

Recently, the correlation between the presence of specific human histo-blood group antigen (HBGA) and the development of NoV infection has been demonstrated, highlighting the relevance of the host genotype as illness factor (Lindesmith L et al., 2003). Interestingly, the virus dose required for causing illness in 50% of cases has been estimated to be close to one to ten NoV particles (Teunis PF et al., 2008). The high infectivity of NoV represents a serious issue for risk management and outbreak prevention.

1.2.5 Epidemiology and transmission

NoVs have been estimated to cause about half of all outbreaks of gastroenteritis and accounting for greater than 90% of viral gastroenteritis worldwide (Patel MM et al., 2009). It has been also estimated that each year NoVs are responsible of 64,000 cases of diarrhea requiring hospitalization, 900,000 clinic visits among children in industrialized countries and up to 200,000 deaths among childrens under 5 years of age (Patel MM et al., 2008). Moreover, the Center for Disease Control and Prevention (CDC) reports NoVs as the most common cause of viral gastroenteritis with an annual occurrence of at least 23 milion infections, the 96% of total cases, in the United States alone with 50,000 hospitalizations and 300 death per year (Mead PS et al., 1999). In a recent CDC's report about the surveillance for foodborne disease outbreaks in 2006, NoVs have been identified as the leading cause of gastroenteritis, accounting for 54% (11,879 cases) of all 1,270 identified outbreaks (621 with a confirmed single cause) from 48 states followed by Salmonella, with 18% of outbreaks corresponding to 3,252 cases (CDC, 2009). In a recent EFSA report, calicivirus has been recognised as the second most common known cause of food-borne outbreaks during 2007, after Salmonella (EFSA Journal, 2009).

Genogroup II NoVs seems to be the most common strain in outbreaks worldwide compared with GI strains. In particular, the GII.4 genocluster has been playing a key role since the mid-1990's, accounting for more than half of all reported outbreaks (Kroneman A et al., 2008; Patel MM et al., 2008). The GII.4 genotype seems to be predominant also in sporadic gastroenteritis in both developing and developed world: in recent investigations it has been

responsible for 68% of pediatric NoV diarrhea cases studied in a nicaraguan city (Bucardo F et al., 2008) and for 81% of pediatric cases reported in an italian city, Palermo, in 2006 (Ramirez S et al., 2009).

The most common route of NoVs infection and transmission during an outbrake is generally the fecal-oral spread (Parashar UD and Monroe SS, 2001) even though the primary source of contamination often comes from food or water. Exposure to aerosols and contact of contaminated environmental surfaces might increase the rapidity and the proportions of NoVs spread in closed settings (Patel MM et al., 2009). NoVs outbreaks have been documented in schools, nursing homes, hospitals, day care centers, hotels and cruise.

NoV outbreaks have been reported and related mainly to water and to a wide range of foodstuff and food commodities such as sandwiches, salads, chiken, oysters, raspberries, cakes and desserts, backery products and others (Baert L et al., 2009).

Four characteristics have been identified to explain the rapid and wide propagation of NoVs spread (Patel MM et al., 2009): i) the low infectious dose of NoVs; ii) the prolonged duration of viral shedding that increases the secondary spread; iii) the NoVs stability in the environment in terms of resistance to chlorine and temperatures ranging from freezing to heating to 60 °C; iv) the lack of complete cross-protection to different virus strains and weak long-term immunity.

1.2.6 NoV persistence in foodstuffs and environment and inactivation methods

The increasing awarness concerning food-borne- and water-borne viruses role in infections and outbreaks have pointed up the need to acquire knowledge about virus persistance in foods and in the environment, in order to set up suitable inactivation methods.

Even if the primary role of the fecal-oral route in food-borne virus transmission has been well enstablished, foods contamination may occur at various stages: contaminated irrigation water or organic-based fertilizer represent a potential pre-harvest contamination source for fruits and vegetables (Carter MJ, 2005); water may be a high risk contamination source also for shellfish (Lees D, 2000). On the other hand, post-harvest contaminations represent the other critical points along the food chain. This risk is mainly related to food handlers (harvester, production plant workers, chefs and caterers) not respecting the hygene regulations and is associated to any food processing procedure that requires manual handling and no heating steps before consumption (Carter MJ, 2005).

Furthermore, the majority of the food-borne viruses, including NoVs, cannot be cultured: this limitation represents one of the biggest obstacle in the evaluation of food-borne presistence,

which often require the employment of cultivable surrogate viruses to collect survival data and inactivation rates. Murin norovirus (MNV) has been frequently used for this purpose in a wide range of food matrices and surfaces: the MNV-1, for example, has been employed to evalutate the inactivation during the production of deep-frozen onions and spinach (Baert L et al., 2008a), in mild-thermal pasteurization of raspberry puree (Baert L et al., 2008b), in the evaluation of sodium hypochlorite and peroxyacetic acid as decontaminating agents for shredded iceberg lettuce (Baert L et al., 2008c), in heat exposure (80 °C) studies (Baert L et al., 2008d), in inactivation evaluations by high-pressure processing (Kingsley DH et al., 2007) and in inactivation studies by chemical biocides on stainless steel (Magulski T et al., 2009). Feline calicivirus (FCV) has also been used for analogous purposes (Whitehead K et al., 2009). Recently, inactivation processes on berries and herbs have been assessed employing HuNoVs and quantitative polymerase chain reaction (PCR) as virus detection method (Butot S et al., 2009). This is a relevant improvement because it overcome the needs of cultivable surrogate viruses; however, it is important to take into account that molecular methods reveal the presence of virus nucleic acids or proteins but do not give information about infectivity. A cell culture model for HuNoVs using a 3-dimensional, organoid model of human small intestinal epithelium has been recently proposed (Straub TM et al., 2007), but the virus replication seems to be modest and the model needs further improvement in order to be effectively employed (Chan MC et al., 2007).

1.2.7 Diagnostics

The failure to propagate NoVs in cell culture has boosted the research to develop effective methods to reveal the NoV presence in both clinical and environmental settings. The cloning of NoV in 1990 (Xi JN et al., 1990) and the consequent analysis on the virus genome have led to a rapid and massive development of a wide range of molecular diagnostic techniques. However, despite almost two decades have elapsed, there is still room for improvement in NoV diagnostics: the tremendous genetic and antigenic diversity among NoV strains (Zheng DP et al., 2006) demands constant update of the current diagnostic methods.

The first technique which has been sucessfully used to detect and identify NoV particles was the immune elecron microscopy (IEM) (Kapikian AZ et al., 1972). The same method and other improved versions, such as the solid-phase IEM (SPIEM), were used to detect NoVs in stool. However, the relative insensitiveness and the needs for highly skilled technicians and huge and expensive equipments have limited the use of the elecron microscopy in large epidemiological and clinical studies (Atmar RL and Estes MK, 2001).

1.2.7.1 *Polymerase Chain Reaction (PCR)*

The complete NoV genome sequencing (Jiang X et al., 1993; Lambden PR et al., 1993) has made possible the employment of Reverse Transcription-PCR (RT-PCR) methods as diagnostic tools for NoV detection and in the last decade, numerous versions of this technology have been developed. The RT-PCR based methods are nowadays considered as the "gold standard" for detection of NoVs in clinical, foods and environmental samples, due to the high sensitivity and the high specificity, the ability to detect genetically diverse NoV strains, the relatively low cost and rapidity.

RT-PCR assays have been developed both for detection (Jiang X et al., 1992; Moe CL et al., 1994; Schwab KJ et al., 1997) and genotyping purposes (Wang J et al., 1994) or in combination with southern hybridisation to differentiate antigenically distinct NoV strains (Ando T et al., 1995). The extensive NoV sequence diversity has quickly led to the needs for broadly reactive assays and considerable efforts have been made to design new primers set (Green J et al., 1995) or primer modifications (Le Guyader F et al., 1996).

In order to increase the sensitivity, approaches based on nested RT-PCR have been used with clinical (Medici MC et al., 2005) and food samples (Häfliger D et al., 1997; Green J et al., 1998). However, despite the gain in sensitivity compared to single-round RT-PCRs, nested RT-PCR presents some limitations due to the additional operations required to perform two consequent PCR rounds which makes this technique more labour-intensive and more prone to cross-contaminations in clinical diagnostic setting (Vinjè J et al., 2003).

Several Real Time RT-PCR formats have also been sucessfully developed, thus allowing to further reduce the time of the analysis, to increase the sensitivity and to semplify the laboratory operations.

Methods based on aspecific reporter dyes like the SYBR green have been applied to NoV detection (Richard GP et al., 2004a; Pang X et al., 2004). These techniques coupled with melting curve analysis showed also the possibility of multiplex analysis targeting several pathogens (Beuret C, 2004) or enabling the differentiation of multiple clusters of NoVs (Richard GP et al., 2004b) simulaneously. A high resolution melting analysis-based method using the ResoLight BRM dye for high -throughput detection and genotyping of NoVs has recently been published (Tajiri-Utagawa E et al., 2009). Furthermore, Real Time RT-PCR based on intercalating dyes, such as SYBR green, has been found to be significantly more sensitive than ELISA methods (Schmid M et al., 2004). The need to improve the assay specificity and add a further confirmatory step in real time analysis led to the introduction of specific probes usually adopting the TaqMan chemistry (Trujillo AA et al., 2006). The

majority of these assays target the most conserved region of NoVs genome, the ORF1-ORF2 junction region, with small differences in terms of primers and probe sequences and level of degeneration employed (Kageyama T et al., 2003; Höhne M and Schreier E, 2004; Jothikumar N et al., 2005; Mohamed N et al., 2006; Ishida S et al., 2008). The introduction of specific probes easily enabled the development of multiplex format assays, for both clinical and environmental samples (Pang XL et al., 2005; Mohamed N et al., 2006; Antonishyn NA et al., 2006; Wolf S et al., 2007). Other real time-based detection methods, such as the light-upon-extension (LUX) real time PCR, have been sucessfully used in NoV detection. (Nordgren J et al., 2008). Recently, the needs for affordable internal controls and for primer and probe standardizations and validations have been indicated as issues of primary importance regarding the use of PCR-based methods in routine diagnostic (Rolfe KJ et al., 2007; Stals A et al., 2009). Despite the needs for further improvements, the high sensitivity, the short time-to-result window, the reduced risk of carry-over contaminations due to the "in-one-tube format" and the ease of use, make the real time PCR methods, the current gold standard in NoV detection.

Further efforts have been made to improve PCR-based assays introducing chemical modifications in order to compensate for high sequence polymorphisms peculiar of NoV genomes. The employment of the universal base inosine in primer design, has been used as a strategy to obtain a broadly reactive PCR method (Green SM et al., 1997). Chemical modifications have been also taken into account for probes design: minor grove binder probes (Höhne M and Schreier E, 2006) and probes based on locked nucleic acid technology (Dreier J et al., 2006) have been used in real time PCR assays. A combination of modified based and minor grove binder-conjugated Eclipse hybridization probes has also been introduced in a one step real time PCR assay in order to overcome design limitations due to nucleotide polymorphisms (Hymas W et al., 2007).

1.2.7.2 *Enzyme Immunoassays (EIAs)*

Various EIA detection methods have been developed for NoV. Despite the high specificity for some NoVs, these assays are generally considered non sensitive enough to detect a wide range of NoV strains (Patel MM et al, 2009). Furthermore, the evaluation of EIA commercial kits showed genotype-dipendent sensitivity and variability according to the circulating strains in the population. The EIA assays are therefore suggested as useful methods for preliminary screening, expecially during gastroenteritis outbreaks, but confirmatory PCR analyses are

reccomended (Dimitriadis A et al., 2006; de Bruin E et al., 2006; Gray JJ et al, 2007). Recently, other immunochromatografic kits have been reported, but they need further improvements to be employed in routine medical use (Khamrin P et al., 2008; Takanashi S et al., 2008; Mutoh K et al., 2009). Rapidity and no needs for highly qualified technicians make the EIA methods suitable for rapid screenings.

1.2.7.3 *Isothermal-based Methods*

Despite RT-PCR has currently regarded as the gold standard for detecting NoVs, new diagnostic methods based on isothermal amplification of the nucleic acids are rapidly gaining popularity. In the last decade, several isothermal based formats and platforms have been developed and sucessfully employed in diagnostic and in basic research. Transcription-mediated amplification (TMA), strand displacement amplification (SDA), self sustained sequence replication (3SR), Nucleic-acid sequence-based amplification (NASBA) and loop mediated isothermal amplification (LAMP) are well established methods, efficiently used as PCR-based systems alternative to address special needs and overcome some intrinsic PCR-based methods limitations. NASBA and LAMP methods have recently been used in NoV diagnostics both in clinical and environmental applications.

NASBA is a transcription-based amplification system performed at constant temperature (usually 41 °C); the continuous amplification of nucleic acids is obtained using a combination of two primers and a mixture of three enzymes, an avian myeloblastosis virus revese transcriptase (AMV-RT), a ribonuclease H (Rnase H) and a T7 DNA dependent RNA polymerase (DdRp). The final product of the reaction consists in a single-stranded RNA amplicon complementary to the target sequence. The reaction can be revealed in Real Time, usually employing a molecular beacon probe or as end-point detection format, by electro-chemiluminescence (ECL) analysis (Deiman B et al., 2002).

NASBA has been sucessfully used in NoV detection in fecal samples (Greene SR et al., 2003; Moore C et al., 2004; Houde A et al., 2006) and foods, such as shellfish (Kou X et al., 2006), ready-to-eat products (Jean J et al., 2004) and oysters (Fukuda S et al., 2008). NASBA has also been employed in environmental analysis of water samples (Rutjes SA et al., 2006a) and swabbed surfaces (Patterson SS et al., 2006). The possibility to design NASBA analysis in a multiplex format (Jean J et al., 2004), the opportunity to overcome some sensitivity problems due to the presence of inhibitory factors sometimes affecting PCR-based assays (Rutjes SA et al., 2006), the short time-to-results, the ability to target single stranded RNA molecules, the

use of a single temperature and the resulting no needs for special thermocycling equipment make this technology particularly suitable as effective alternative to PCR in NoV detection.
LAMP is an isothermal gene amplification technique which employs a set of six specially designed primers and a DNA polymerase with strand displacement activity. All the procedure is completed in less than one hour and the amplification products can be monitored in real time in an inexpensive turbidimeter or detected in end-point systems by agarose gel electrophoresis or by naked eye using appropriate dyes (Parida M et al., 2008).
A LAMP system has been developed for NoV detection in fecal specimen and a NASBA/LAMP combined method has been used for NoV detection in foods (Fukuda S et al., 2008). Recently, a LAMP-based commercial kit (Iturriza-Gómara M et al., 2008) and a modified version, improved for GI detection (Yoda T et al., 2009), has been evaluated in NoVs diagnostics with good results.

1.2.7.4 *Microarrays*

The current methods for the differential identification of virus strains are based on PCR amplification and amplicons sequencing. NoV genotyping is particularly important for outbreaks monitoring and control, but no consensus about harmonization of genotyping assays has been reached at international level (Mattison K et al., 2009). Morover, sequencing methods are considered precise for identification, but a certain lack of sensibility for mutant detection in mixed population, composed simultaneusly by several different strains, might introduce a bias in favor of dominant variants. In addition, the impossibility to analyze more than one target per reaction makes the sequencing-based techniques limited in terms of high-throughput capability and quite expensive for screening purposes. In order to overcome these limitations, some authors suggested the use of microarray analysis for NoV detection. A tiling microarray bearing 13,000 elements for the specific detection and characterization of common foodborne viruses, including NoVs, has been recently developed (Ayodeji M et al., 2009). A generic tag array to detect NoVs in water has also been developed to overcome the difficulties in environmental samples sequencing due to co-amplification of non-specific cDNAs (Brinkman NE and Fout GS, 2009). A microarray based on the hybridization of long PCR products (917 bp), that encompass two major regions currently employed for the analysis of NoV genome, has been proposed as strategy to solve a problem coming from the post PCR sequencing: the use of short amplyfied products, often targeting different sequences result in difficult comparisons and virus tracking among research laboratories (Pagotto F et

al., 2008). NoVs and Astroviruses have been also detected and genotyped in fecal samples using a microarray-based strategy (Jääskeläinen AJ and Maunula L, 2006).

1.2.8 Detection of NoV in foods, surfaces and waters

Since foods is one of the most important vector of NoV transmission, significant efforts are underway to improve and develop new methods for NoV recovery and detection in foods.
Several diagnostic approaches have been already published for a wide range of food matrices: molluscs such as oysters (Le Guyader FS et al, 2009; Gentry J et al., 2009) and blue mussels (Comelli HL et al., 2008), fruits and vegetables such as raspberries (Le Guyader FS et al., 2004; Rzezutka A et al., 2005; Butot S et al. 2009; Kim HY et al., 2008; Morton V et al., 2009), strawberries and lettuce (Cheong S et al., 2009a), green onions (Guévremont E et al., 2006), basil, mint and parsley (Butot S et al. 2009; Kim HY et al., 2008; Morton V et al., 2009), grapes (Kim HY et al., 2008). Detection methods have also been developed for cheese (Fumian TM et al., 2009), whipped cream (Ruties SA et al., 2006b), deli ham (Morton V et al., 2009), ham, turkey and roast beef (Schwab KJ et al., 2000), mixed lettuce, fruit salad, penne and tagliatelle salads (Baert L et al., 2008e).
One of the most challenging aspect in virus recovery and detection from foods is due to the wide range of chemical and physical characteristics peculiar of the differtent food matrices, demanding for optimized methods for each food. In the previously published studies, consistent efforts have been spent in the optimization of the elution, the concentration and the RNA extraction procedures, comparing different methods for each food. What has emerged from these studies is the need for an optimized combination of elution-concentration-nucleic acid extraction steps depending on the analyzed food; furthermore, the best approach to effectively detect viruses in foods is often different for different food matrices (Rutjes SA et al., 2006b).
The needs for affordable methods for virus recovery and analysis from contaminated surfaces is another foundamental task in order to track outhbreaks and develop effective confirmatory assays. Recently, environmental swabs have been proposed as a useful tool in NoV diagnostics in outbreak settings (Boxman IL et al., 2009). In the same way, swabs taken directy from hands have been successfully used to study the route of viruses transmission in outbreak settings (Boxman I et al., 2009).

The surveillance of viral pathogens in water is another important task for several reasons: the evaluation of the incidence and the behaviour of viruses and the risk of infections, the

evaluation of the efficiency of treatment and disinfection processes, water quality monitoring and compliance of water with specifications and guidelines, the study of the epidemiology of waterborne viruses. One of the most difficult aspect in the virological analysis of water is the development of an effective concentration process, required to recover low number of virus particles in large volume of water (Bosch A et al., 2008). Numerous methods have been employed to achieve this difficult task (Wyn-Jones P, 2007) but no single method seems to meets all the desired criteria.

Several strategies for NoV concentration and detection from water have been published (Jones TH et al., 2009; Haramoto E et al., 2009; Albinana-Gimenez N et al., 2009; Karim MR et al., 2009; Skraber S et al., 2009; Victoria M et al., 2009; Brinkman NE and Fout GS, 2009; Cannon JL and Vinje J, 2008; Lambertini E et al., 2008; Laverick MA et al., 2004; Beuret C, 2003; Fout GS et al., 2003; Gilgen M et al., 1997) and used to analyze urban surface waters (Aw TG et al., 2009; Skraber S et al., 2009; Miagostovich MP et al., 2008; Pusch D et al., 2005), tap water (Haramoto E et al., 2005;), groundwater (Petrinca AR et al., 2009; Cheong S et al., 2009b), estuarian (Hernandez-Morga J et al., 2009) and marine costal water (Asahina AY et al., 2009; Katayama et al., 2002;), raw sewage (Nordgren J et al., 2009; Kitajima M et al., 2009; Katayama H et al., 2008; da Silva AK et al., 2007; La Rosa G et al., 2007; Lodder WJ and de Roda Husman AM, 2005), treated sewage (Nordgren J et al., 2009; Kitajima M et al., 2009; Katayama H et al., 2008; da Silva AK et al., 2007; Lodder WJ and de Roda Husman AM, 2005), river water (Hamza IA et al., 2009; Cheonghoon L and Sang-Jong K, 2008; Westrell T et al., 2006; Haramoto E et al., 2005; Lodder WJ and de Roda Husman AM, 2005) and bottled water (Liu J et al., 2007; Butot S et al., 2007; Lamothe GT et al., 2003; Gassilloud B et al., 2003; Beuret C et al., 2002).

1.3. PEPTIDE NUCLEIC ACIDS

1.3.1 Introduction

Efficient targeting of nucleic acids both for diagnostic and therapeutic purposes is currently one of the most relevant task in molecular biotechnology. The development of synthetic compunds able to interact with nucleic acids in an effective and sequence-specific manner is the goal of numerous research groups. Several nucleic acid analogs and mimics have been designed and developed to overcome limitations of natural nucleic acids, to ease the process of oligo syntesis, to improve affinity and selectivity, to increase nuclease resistance, to improve the thermodinamic properties and add functionalities like the ability to cross cell membranes or the ability to provide fluorescence signals. Some nucleic acid analogs have been syntesized introducing modifications or substitutions at the phosphodiester backbone level like the phosphothioates and the N3'-P5' phosphoramidate DNA. Others have been modified at the nucleobase/sugar ring positions like the morpholino-DNA or the locked nucleic acids (LNA). At the beginning of 1990s, a class of DNA analogs with a very peculiar chemistry, in which both the type of bond between the nucleotide units was changed and the sugar moiety removed in favour of acyclic structures, was proposed to the scientific community: the Peptide nucleic acids (PNAs) are nowadays one of the most interesting class of these new DNA analogs.

The first aminoethylglycine-based PNA was invented first reported by a danish team leaded by Peter Nielsen (Nielsen PE et al, 1991; Buchardt O et al, 1992). PNAs were originally concieved and designed as a ligand for recognition of double stranded DNA (dsDNA) via Hoogsteen base pairing in the major groove, in analogy to triplex-forming oligonucleotides. In this nucleic acid analogs, the negatively charged deoxyribose- or ribose-phosphate backbone is replaced with a polyamide of N-(2-aminoethyl)glycine that provide an uncharged scaffold, bearing the nucleobases attached via a methylene carbonyl linker (Figure 1.3).

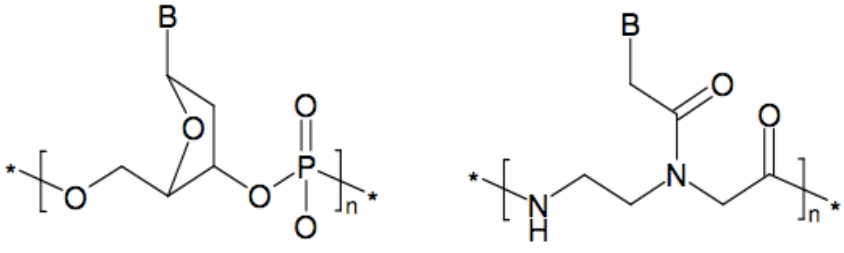


Figure 1.3. Schematic representation of DNA (*left*) and PNA (*right*) structures

This new class of molecules revealed soon unique physicochemical and biochemical properties, making PNAs valuable tools for reseach, diagnostics and medicine.

1.3.2 Synthesis

The syntetic process of PNAs has its basis in the synthesys of peptides, and borrows from this field all the main assembly steps (Figure 1.4). The solid phase syntesis, originally developed by Merrifield in the sixties (Merrifield B, 1963), is currently the more common approach in PNA production. The crucial step in the whole process, the elongation of the PNA, is driven by the condensation of the single building block-carboxy function with the deprotected amino function of the growing chain. Usually, the overall assembly workflow consists of several cycles of deprotection, activation, coupling and capping. These repeated steps are preceded by the preparation and the binding of the first monomer to an appropriate solide support, such as polystirene beads carring proper reactive chemical groups, like the 4-methyl benzhydrylamine groups (MBHA resin). The last operation, consists in the cleavage of the completed PNAs from the resin, in order to further characterize, purify and employ the PNAs for specific purposes.

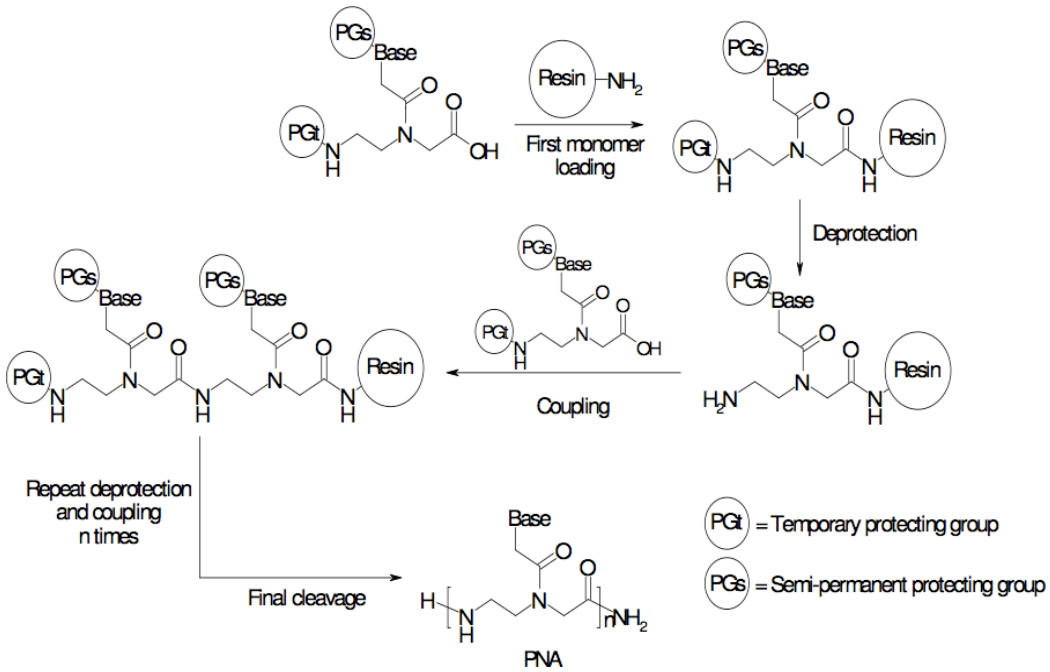


Figure 1.4. Generic scheme for solid phase synthesis

Two main strategies are widely empolyed nowadays in PNAs synthesis, according to the chosen monomer protecting groups: the tert-butyloxycarbonyl (Boc) and fluorenylmethyloxycarbonyl (Fmoc) solid phase approaches. Moreover, these two strategies have been successfully adapted for automated solid pahse synthesis using commercial peptide synthesizers.

The first reported PNA, was a T_{10} oligomer assembled using the Boc strategy. Later, when mixed sequence have been considered, the protection of the exocyclic amino groups of the bases soon became essential in order to avoid the side reactions involving the nucleobasis. The introduction of the benzyloxycarbonyl (Z) protector group is commonly employed to accomplish this task in the Boc strategy, thanks to the differential acid lability between the two groups.

The Boc group is removed in acidic conditions using Trifluoroacetic acid (TFA) and a scavenger like m-cresol is added to prevent the alkylation on the nucleobases due to the t-butil cations released during the deprotection step.

The monomer activation, that enables the formation of the active esters, is usually performed in the presence of peptide coupling reagents like the 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), or the 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and diisopropylethylamine (DIEA). The coupling takes place using a proper combination of solvents like the combination of N-methylpyrrolidone (NMP) with pyridine or dichloromethane (DCM). The last step at the end of each elongation cicle, consist in the capping of oligmer that fails to extend using for example a solution of acetic anhydride and pyridine.

After the complete synthesis, which occours in the elongation scheme in the C- to N-term orientation, the Boc/Z protected oligomer is cleaved from the resin using a strong acid such as the trifluoromethane sulphonic acid (TFMSA).

The Boc strategy is well established and it is probably the mostly used approach in PNA synthesis. However, a notable limitation of this technique, is due to the lability of some labeling molecules or conjugates in the acidic conditions used for the deprotection/cleavage scheme of the Boc chemistry. In order to overcome this major drawback and employ milder and more compatible chemical conditions with certain molecules needed for PNAs derivatization, the Fmoc approach have been successfully employed. This synthetic strategy usually exploits the benzhydryloxycarbonyl group (Bhoc) for the protection of the exocyclic amino group of the nucleobases A, C and G and differs from the Boc strategy mainly for the removal conditions of the protecting groups: in the Fmoc/Bhoc synthesis, the removal of the

Fmoc group is carried out in alcaline conditions, using for example a solution of piperidine in dimethylformamide (DMF), whereas the final Bhoc deprotection and the cleavage from the resin is performed in acidic conditions using TFA with the help of a scavenger like the m-cresol in order to prevent the alkylation of the nucleobases by cations generated during the TFA treatment.

Although the Boc and Fmoc approaches are the mostly used synthetic strategies, researchers are still looking for novel PNA synthetic strategies to improve performances, simplify the synthetic processes or solve specific problems. A recent example using the benzothiazole-2-sulfonyl (Bts) as an amine-protecting group has been proposed (Lee H et al., 2007); this strategy relies on the use of self-activated cyclic Bts PNA monomers, where the Bts group plays a double role: as protecting group of the amine in the PNA backbone but also as activating group for the coupling reaction; this process seems to provide extremely pure PNA oligomers without requiring any further coupling reagents.

1.3.3 Properties of PNAs

The peculiar structure of PNAs consists of nucleobases attached via methylene carbonyl linkages to the acyclic, achiral and neutral backbone made by repeated units of N-(2-aminoethyl)-glycine; this structure allows the formation of specific hybridization between PNA and cDNA or RNA due to the similar intramolecular distances and nucleobase configuration available in the natural nucleic acids.

The determination of PNA-DNA and PNA-RNA duplexes by NMR investigation (Eriksson M and Nielsen PE, 1996; Brown SC et al., 1994) and the analysis of PNA/DNA and PNA/PNA duplexes by X-ray christallography (Rasmussen H et al., 1997; Betts L et al., 1995; Menchise V et al., 2003) have further elucidated the PNA behaviours upon hybridization, providing valuable information about the adopted conformations in different complexes: the DNA/PNA duplex is close to the B-form conformation whereas the RNA/PNA duplex adopts a conformation close to the A-form. PNAs are also able to adopt peculiar conformations which differ from the other nucleic acid helices: a distinct conformation, defined P-form, seems to be common in the 2PNA/DNA triplex formation; furthermore, a wide helix, with almost twice the pich of a common A- or B-form helix has been observed in the PNA/PNA duplex (Figure 1.5). These structural details provide remarkable examples about the exterme flexibility of the PNAs hybridization properties.

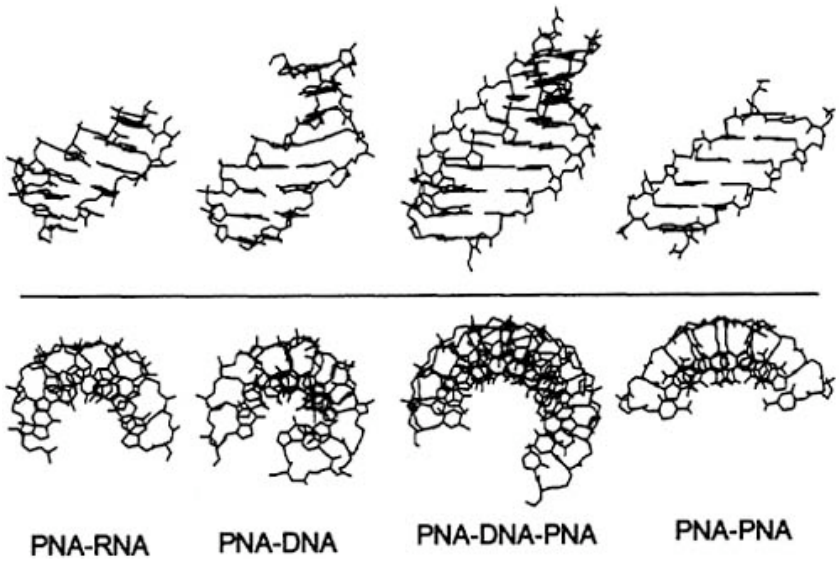


Figure 1.5. Structures of PNA complexes (Nielsen PE, 2004)

The uncharged nature of PNAs is one of their more distinctive character, responsible for the stronger binding and the better thermal stability of PNA/DNA duplex compared with the analogous complexes made only by DNA strands. As further consequence of this property, single-base mismatches have usually a more drastic destabilizing effect than in a DNA/DNA duplex; hovever the magnitude of this effect is not constant along the oligomer sequence since a certain correlation between mismatch position and duplex destabilization has been observed (Igloi GL, 1998). These features play a key role and help in the design of highly specific diagnostic methods.

The lack of repulsion between PNAs and hybridized nucleic acids and the consequent no needs for positive ions to counteract the interstrand repulsion, that usually occurs between conventional nucleic acids upon duplex formation, allow for the efficient hybridization of PNAs to DNA molecules at low ionic strength. Low-salt conditions may be important to destabilize intramolecular folding structures in RNA and DNA molecules, thus making sequences more accessible to complementary PNAs. This might be extremely useful to improve diagnostic performances with particularly difficult and inaccessible sequences. PNAs are also stable across a wide range of pH and exhibit good performances in the presence of organic solvents (Sen A and Nielsen PE, 2007).

The unnatural backbone is also responsible for the high biostability: the resistance to nucleases and proteases (Demidov VV et al., 1994) make the PNAs particularly attractive for *in vivo* diagnostics and along with the peculiar hybridization abilities, as antisense and anti-gene agents.

The neutral nature of PNAs, however, is sometimes source of drawbacks: one major limitation is the poor water solubility compared to DNA; the sequence composition (purine:pyrimidine ratio) and the oligomer length are key factors in determining the PNA solubility (Hyrup B and Nielsen PE, 1995). Aggregates of single stranded PNAs have been observed using ^{1}H NMR analysis (Leijon M et al., 1994) and self-melting processes in aqueous solution have also been reported (Tomac S et al., 1996). Conjugation with cationic ligands, both *via* N/C terminus- or backbone- modification strategies, has been used in order to improve the PNA solubility (Kumar VA and Ganesh KN, 2006). Another important feature of single stranded PNAs consists in the ability to interact with double stranded DNA (dsDNA) in several configurations, depending on sequences and conditions (Figure 1.7).

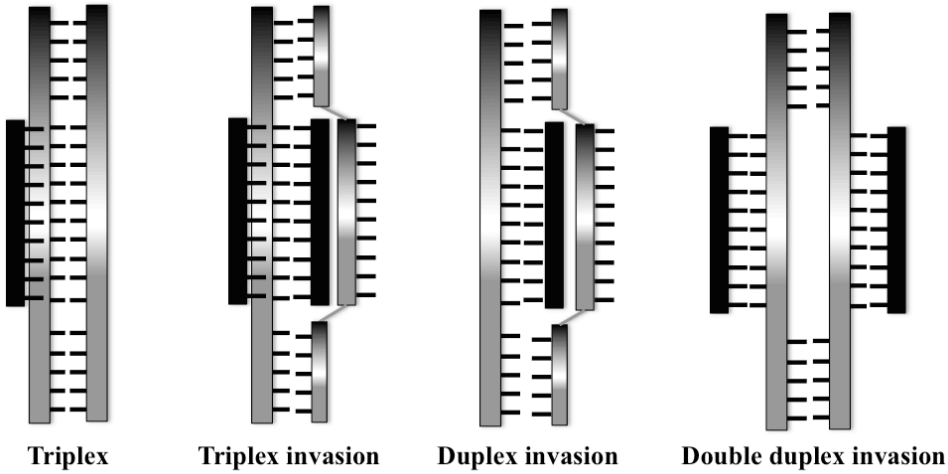


Figure 1.7. Complexes formed between PNA and double-stranded DNA

Usually, the hybridization process is driven by a strand-invasion event, a mechanism by which the two complementary DNA strands are locally dissociated and the binding of the PNA oligomer(s) to one or both the exposed strands occurs, depending on the sequence characteristics. The duplex and the triplex invasion mechanisms are the most common modes to break up the double helix of DNA, targeting a specific region with PNAs.

The triplex invasion occurs when two homopyrimidine PNAs bind the complementary DNA sequence involving both Watson-Crick and Hoogsteen hydrogen bonds interactions, forming a very stable triplex; in this configuration, the displaced non-compementary DNA strand forms a peculiar single-stranded conformation named D-loop. In order to improve PNA ability to perform triplex invasion, the so-called bisPNAs have been conceived. These PNAs consist of palindromic homopyrimidine sequences joined by a flexible linker in the middle

bearing some chemical modifications, such as the cytosine replacement by pseudoisocytosines (J bases) in the PNA strand forming Hoogsteen bonds. The bisPNAs usually exhibit high melting temperatures (T_m) and may have superior binding efficiency compared with mono-PNAs. On the other hand, duplex invasion may occur targeting homopyrimidine DNA sequences. In this mechanism, a single PNA interacts with DNA via conventional Watson-Crick bonds displacing the non-complementary DNA strand. However, this kind of complexes seem to be quite labile and mixed sequences need further chemical modifications and specific conditions to open and bind a dsDNA targets via duplex invasion. Another kind of PNA:DNA:DNA complex may occur using cytosine-rich PNAs: in this tripex structure, the PNA interact with the dsDNA forming Hoogsteen bonds.

In order to overcome the sequence constrains of duplex and tripex invasion, the double-duplex invasion employing two pseudocomplementary PNAs (pcPNAs) has been proposed. In this configuration, the dsDNA is locally opened and each strand interact with the complementary PNA, involved in Watson-Crick base pairing. Since the formation of extremely stable PNA:PNA complexes would be dominant without any further chemical modifications, the tymine and adenine have been replaced by thiouracil (sU) and diaminopurine (D) respectively in order to sterically destabilize such complexes.

1.3.4 Chemical modifications of PNA: backbone and nucleobase modifications

Numerous rationally modified PNA analogues have been proposed attempting to improve some characteristics and overcome some current limitations in standard PNA properties. The major limiting issues concerning practical PNA applications can be generally classified as: i) poor aqueous solubility which can limit the applications of PNAs in diagnostic and terapeutic fields and it has been minimized employing conjugation with cationic ligands at the N/C terminus of the PNA, such as polyamines and lysine, or incorporation of a guanidine functional group into the PNA backbone; ii) poor cell penetration abilities, that limit the *in vivo* diagnostic and terapeutic applications, have been improved by conjugation with a wide range of transfer molecules. Strategies based on cell-penetrating peptides are among the mostly employed in order to improve delivery of therapeutic molecules, including PNAs (Heitz F et al., 2009); iii) DNA/RNA binding limitations: the ability of PNAs to bind DNA/RNA in parallel and antiparallel orientations, might negatively affect therapeutic applications, through a reduction in target specificity leading to side-effects due to interactions with other genomic sequences.

The introduction of chirality into the PNA backbone has been used to address this limitation adding orientational selectivity properties to the modified PNAs. The same approach has been employed to improve the mismatch recognition ability of PNAs; iv) poor selectivity for DNA vs RNA binding: although the hybridization of PNA to DNA or RNA molecules bearing identical sequences may present some T_m differences, it has been shown that it is possible to trigger a shift in the equilibrium towards the desired complex with a rational design using conformational preorganization strategies: ciclohexyl (Govindaraju T et al., 2003; 2004a) and ciclopentyl (Govindaraju et al., 2004b; 2004c) PNAs are effective examples of these approaches.

Other PNA modification strategies have been focused on the replacement of conventional nucleobases with "non-Watson-Crick" nucleobases or other molecules that act as analogs but introduce some peculiar functionalities. Modifications at the nucleobases level have been investigated attempting to: i) fine-tuning some hybridization or base-base interaction properties: examples of these types of nucleobases are the pseudoisocytosine commonly employed in bis-PNAs (Egholm et al., 1995), the previously mentioned diaminopurine (Haaima G et al., 1997), used in place of adenine to improve the T_m, or the thiouracil used in the pcPNAs (Lohse J et al., 1999); ii) affect binding affinity and/or add extra-features. Modified nucleobases substitutions have been used in PNAs to increase the binding affinity (Wojciechowski and Hudson RH, 2009), introducing elements acting as universal nucleobase (Hirano T et al., 2009; MacKinnon KF et al., 2007) or add fluorescence features to the PNA molecules (Köhler O and Seitz O, 2003; Bethge L et al., 2008).

1.3.5 Thiazole orange-conjugated PNAs: an example of backbone and nucleobase modification

Development of probes, that provide detectable fluorescence emission signals upon hybridization and simultaneously exhibit high sensitivity and specificity, is one of the most investigated field of reseach. These kinds of molecular tools, are essential for the development of effective nucleic acid detection methods; molecular beacons (Tyagi S and Kramer FR, 1996) and TaqMan probes (Holland PM et al., 1991) are among the most well-enstablished and effective class of probes for homogeneous DNA and RNA detection. However, assays involving these kinds of probes might sometimes result difficult to optimize for particular applications such as SNPs analysis, RNA detection in living cells or assays suitable to target very short or "difficult" sequences, characterized by stable secondary structures or repeated stretches of nucleotides bearing the same sequence. Consistent efforts

have been made during the last decade to investigate the possibility to couple suitable fluorescent systems with PNAs in order to exploit their oustanding hybridization properties in fluorescence-based nucleic acid detection assays and overcome some limitations associated to the conventional DNA probes. The wide spectra of derivatization possibilities offered by various PNAs and the relative ease in derivatize PNAs both at the nucleobase and backbone level have been investigated in several works in order to obtain fluorescent PNA probes suitable for homogeneous DNA and RNA detection assays. Tiazole Orange (TO) conjugation has been shown to be one of the most effective strategy to achieve this objective. Published almost simultaneously by two different research groups (Seitz O et al., 1999; Svanvik N et al., 2000), two different TO conjugation approaches have been proposed, attempting to pursue partly different objectives. These two strategies represent two different derivatization concepts and strategies: i) theter the TO at one PNA terminus using a linker, letting the dye free to fold-back binding the PNA-DNA duplex, or to interact with the protruding single-stranded portion of the hybridized nucleic acids (Figure 1.7). This approach, named Light-Up by its inventors, have been developed to provide highly specific probes to be used in homogeneous assays (Svanvik N et al., 2000). Applications using real time PCR have been developed and kits for citomegalovirus (CMV) and Epstein-Barr virus (EBV) detection have also been commercialized under the name of ReSSQ® kits; ii) replace one nucleobase with a TO molecule forcing the dye to interact with nucleic acids exclusively upon hybridization with the complementary target sequence (Figure 1.8). This strategy has been mainly desinged for SNPs recognition and relies on the local perturbations that occur when a mismatched base pair is localized near the TO molecules (Seitz O et al., 1999).

The Kubista's group have covalently linked the TO using different linkers and attaching the PNAs either to the quinoline or to the benzothiazole nitrogen of the dye (Svanvik N et al., 2000). Several Light-Up with different sequences (mixed and homopyrimidine) and length have been characterized and studied. For all the probes but one, the hybridization with the target sequence results in an increase in dye fluorescence accompained by a red-shift of the dye absorption band. However, the magnitude of the fluorescence enhancement (from 2- to 45-fold), along with the red-shift and the free probe fluoresence behaviours resulted extremely different among the probes, depending on the sequence composition. Homopyrimidine probes showed low free probe fluorescence while large variations have been observed for the mixed sequences. Moreover, one homopyrimidine probe expected to form a $(PNA)_2$-DNA triplex showed a good sensitivity in the presence of a single-mismatch oligonucleotide and a good fluorescence enhancement upon hybridization with a PCR

product; however, the probe performances in the presence of PCR products resulted lower if compared with the analogous oligonucleotide experiments (Svanvik N et al., 2000).

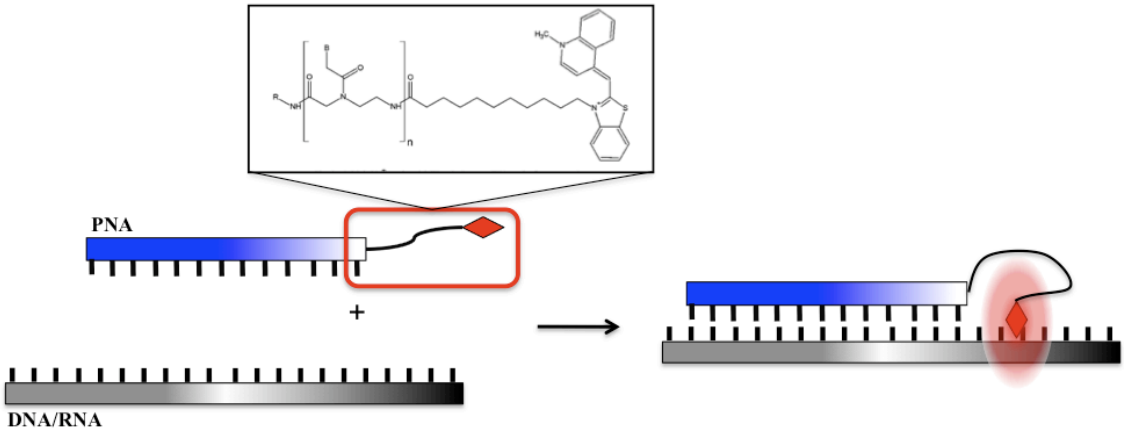


Figure 1.7. Schematic representation of LightUp probe. The TO is thetered at one PNA terminus using a linker. The dye interacts with the PNA-DNA duplex increasing the fluorescence emission

The Light-Up probes have been applied to detect post-PCR products of the human b-actin gene and the *invA* gene of *Salmonella* with high resolution of a single mismatch (Isacsson J et al., 2000). Assays for the *gusA* gene of the *Escherichia Coli* ß-glucuronidase (Svanvik N et al., 2000b) and for part of the plasmid-borne virulence gene *yadA* (Wolffs P et al., 2001) have also been developed. TO-modified PNA oligomers have also been successfully used in clinical diagnostic applications (Wirgart BZ et al., 2005; Leijon M et al., 2006) and the same technology has been implemented in commercial kits under the name of ReSSQ® line (Wirgart BZ et al., 2005).

The Seitz's group has devised the Forced Intercalation (FIT) probes in which the TO replaces an internal nucleobase; the main advantage for this TO-conjugation approach is provided by the responsiveness of the probes to structural perturbations such as those imposed by single base mismatches. Moreover, TO linked to the PNA backbone *via* a carboxymethylene spacer was found to pair equally well with the four canonic nucleobases acting as a universal base, maintaining the duplex stability (Köhler O and Seitz O, 2003). The binding geometry of the TO molecule have been extensively investigated by the same research group, linking both the benzothiazole and the quinoline ring to the PNA backbone with carboxyalkyl spacers of varying length (Köhler O et al., 2005). TO linked through an acetyl theter to the quinoline ring has been shown to be the most effective configuration in terms of thermal stability and fluorescence performances. Moreover, this kind of probe showed mismatch recognition capabilities even at room temperature (25 °C) which increase at higher temperatures (60 °C).

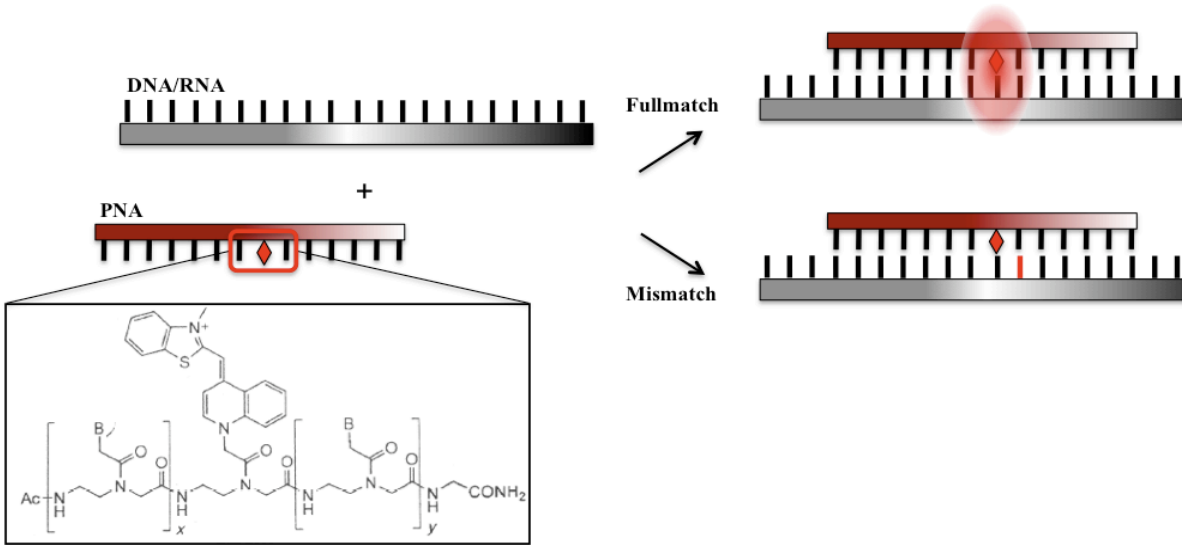


Figure 1.8. Schematic representation of FIT probe. A nucleobase is replaced with a TO molecule forcing the dye to interact with nucleic acids upon hybridization with the complementary target sequence. The fluorescence emission, increase in the presence of the fullmatch sequence allowing the detection of mismatch

Also the optical properties of the FIT probes have been extensively investigated (Jarikote DV et al., 2007). Recently, an application of this probes in a real time PCR assay has been reported: FIT probes have been used for the unambiguous detection of two SNPs in the cystic fibrosis transmembrane regulator (CFTR) gene. In this report, a new class of probes composed by D-ornithine rather than aminoethylglycine as PNA backbone, have been employed in order to improve the intensity and the specificity of fluorescence signaling (Socher E et al., 2008a).

Several fluorescent base surogates have been introduced in PNA probes such as flavin (Ikeda H et al., 2001), psoralen (Okamoto et al., 2001), naphthalimide (Ikeda et al., 2002) which, however, led to a significant duplex destabilization: TO-conjugation to PNAs is one of the most succesful strategies to provide a fluorescent universal base for PNA-based probes to be used in real time homogeneous assays.

1.3.6 Other examples of PNA modifications for research and diagnostic applications

Beside the efforts made by chemists to improve PNA performances such as sequence specificity, solubility and cell permeability, the interest for the use of PNAs as probes for both in *vitro* and in *vivo* analyses is rapidly increasing. However, in order to be able to benefit from the outstanding PNA properties in diagnostic applications, this kind of oligomers needs to be

modified in order to provide a reporter signal suitable to be detected with the instrumentations usually available in diagnostic and research laboratories. PNA-based molecular beacons are an effective example of PNA probes (Figure 1.9). Molecular beacons are probes originally made linking a fluorophore (F) and a quencher (Q) group at the opposite ends of a standard DNA oligonucleotide (Tyagi S and Kramer FR, 1996). These probes are designed introducing complementary terminal DNA sequences that enable the probe to fold into a hairpin conformation, thus bearing the F and the Q in close proximity. As long as the probe mantains this conformation, the fluorescence is quenced whereas upon probe hybridization the distance between Q and F is enough to restore the fluorescence. Molecular beacons are particulartly suited for applications in nucleic acid amplification assays or RNA analysis in living cells. The first reported example of PNA beacon consisted in a surface immobilized chimeric PNA-DNA probe used to detect PCR products in a microtiter-well format (Ortiz E et al., 1998). The hybrid PNA-DNA was rapidly abandoned in favour of an all-PNA approach, in which the F and the Q are bound to the side chain of additional aminoacid residues such as cysteine or to modified nucleobase building blocks (Seitz O, 2000).

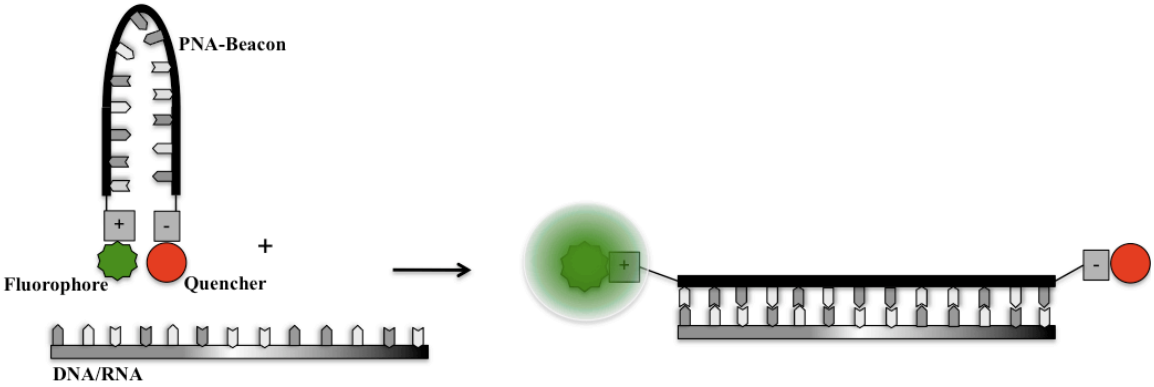


Figure 1.9. Schematic representation of a stemless PNA-Beacon

A notable characteristic of PNA-based beacons is that there is no need for hairpin structures employed to quench the fluorescence in DNA beacons, because the neutral backbone and the hydrophobic structure of PNAs seem to be sufficient to favour a condensed structure which brings the Q suffinciently close to the F to assure a quenched-state probe. These stemless PNA beacons ease the sequence design phase removing the constrain due to the introduction of complementary sequences in the probe. Moreover, PNA beacons have been used to detect nucleic acids in various buffers and protein-containing solutions (Kuhn H et al., 2001; 2002a) being less sensitive to the presence of DNA-binding proteins and less affected by salt

concentrations than DNA beacons. PNA-beacons have been used for recognition of specific sequences in dsDNA with the aid of PNA openers (Kuhn H et al., 2001; 2002b), for SNP detection in the exon 6 of the XPD gene using real time PCR (Petersen K et al., 2004), to improve detection and quantification of rRNA in solution and in whole cells also in combination with flow cytometry (Xi C et al., 2003), as reporter signal in real time monitoring of branched rolling-circle DNA amplification (Smolina IV et al., 2004), for mismatch recognition up to >90 : 1 signal discrimination using ion exchange HPLC (Totsingnan F et al., 2008). Recently a new low-noise stemless PNA beacon has been proposed: in this approach, the intercalator dye thiazole orange is introduced as base surrogate in the probe and used as donor for FRET and the terminally appended near-infrared (NIR) dye NIR667 serves as acceptor dye. These new class of PNA beacons have been shown to be amongst the most sensitive hybridization probes enabling also mismatch recognition at low temperatures, thus extending the temperature range for SNPs recognition, compared to traditional beacons (Socher E et al., 2008b).

1.3.7 PNAs in diagnostic applications

1.3.7.1 PNA Fluorescence *in situ* hybridization (FISH)

A wide range of FISH-based techniques has been successfully employed in clinical and environmental diagnostics for targeting specific DNA and RNA sequences in tissues, eucariotic cells, bacteria, yeasts and viruses (Volpi EV and Bridger JM, 2008). FISH methods rely on local hybridizidation of specific probes that bind the target sequences inside cells (*in situ*), thus allowing a sort of spatial-localized single-cell analysis. In order to be analyzed, FISH probes are usually labelled with fluorophores or linked to enzymes which convert a substrate (i.e. fluorogenic molecules) to some detectable signal. Probes and targets are visualized *in situ* by microscopy techniques. FISH-based assays have been developed for gene mapping, chromosomial abnormality identification, diagnosis of bacterial and virus disease, analysis of genetic markers, tumor cytogenetic tests, abnormal gene expression. FISH can also be employed in research for mapping chromosomial genes, studying the genomes and their evolution, analyzing the cell organization and the RNA expression.

The use of PNAs as FISH probes enabled to obtain some important improvements, opening new possibilities in this field: the higher sensitivity and specificity of PNAs, compared with DNA probes, result in better signal-to-noise ratios with consequent lower signals background.

Sometimes, these characteristics permit to improve also the speed, the stability and the reproducibility of the assays and even single point mutations are detectable. Moreover, the most informative targets are often embedded in poorly acccessible sequence contexts for standard DNA probes: PNA-FISH showed better results targeting scarcely accessible sequences such as GC-rich telomeric regions, or ribosomial RNA (rRNA) variable regions often characterized by extremely stable intramolecular secondary structures (Wilks SA and Keevil CW, 2006).

PNA-FISH was first proposed to mesure individual telomer lengths of chromosomes during the metaphase (Lansdorp PM et al., 1996). Several molecular cytogenetic applications have been therefore developed: quantitative telomere analysis on intherphase cells (de Pauw ES et al., 1998), analysis of chromosomic aberrations (Flaquè MC et al., 2006; Boei JJ et al., 2000; Deng W and Lucas JN, 1999; Boei JJ et al., 1998), single base discrimination in the satellite repeat structure in mammalian cells, aneuploidy assessment in human sperm (Pellestor F, 2006). The high discrimination properties of PNAs have also been used for chromosome identification, targeting chromosome-specific repeat sequences like the interspersed alpha-satellite sequences (Chen C et al., 2000) also in multiplex format (multicolor FISH) labelling the probes with different fluorophores (Taneja KL et al., 2001). The application of PNA-FISH in noninvasive prenatal diagnosis has also been successfully demonstrated by detecting gamma-globin mRNA in fetal nucleated red blood cells from maternal blood (Larsen RD et al., 2003).

PNA-FISH finds also wide application in microbiology for both diagnostic and research purposes. Several bacteria, yeasts and viruses have been detected in clinical and environmental settings. Moreover, PNA-FISH techniques have been shown to be effectively adaptable to a wide range of different matrices. The PNA chemiluminescent in situ hybridization (CISH) has been employed to detect *E.Coli* in liquid cultures (Perry-O'Keefe H et al., 2001), water (Prescott AM and Fricker CR, 1999; Stender H et al., 2001; Stender H et al., 2000), water-associated biofilms (Lehtola MJ et al., 2007), blood cultures (Søgaard M et al., 2005). *Mycobacterium immunogenum* has been detected in sputum and metalworking fluids (Selvaraju SB et al., 2008) thus showing the possibility to develop diagnostic systems both for clinical and industrial settings. Other *mycobacterium* spp. have been detected and differentiated with specific PNA-based ISH probes in water-associated biofilms (Lehtola MJ et al., 2006; Lehtola MJ et al., 2007), biopsis from infected humans or animals (Lefmann M et al., 2006), formalin-fixed paraffin-embedded archival biopsy and autopsy samples (Zerbi P et al., 2001), solid cultures and sputum (Hongmanee P et al., 2001) and linquid cultures (Padilla

E et al., 2000; Drobniewski FA et al., 2000; Stender H et al., 1999). Numerous applications for several *candida* spp. have been developed (Oliveira K et al., 2001; Rigby S et al., 2002; Søgaard M et al., 2005; Wilson DA et al., 2005; Reller ME et al., 2007; Shepard JR et al., 2008; Bishop JA et al., 2008) allowing to reduce the time of identification (Gherna M and Merz WG, 2009) and the costs for clinical treatments (Forrest GN et al., 2006; Alexander BD et al., 2006). PNA FISH has been used also to investigate specific issues such as the influence of physico-chemical parameters (Gião MS et al., 2009a) and chlorination stress (Gião MS et al., 2009b) on *Legionella pneumophila* survival in drinking water biofilms or applications in the food industry such as the identification of *Brettanomyces* isolates from wine (Stender H et al., 2001) or the detection of *Cronobacter* genomospecies in powdered infant formula (Almeida C et al., 2009). PNA-ISH has been applied also to the detection of viruses such as the Parvovirus B_{19} (Bonvicini F et al., 2006) enabling the quantitative detection in single infected cells (Bonvicini F et al., 2007) and HIV (Hagiwara et al., 2006). Combinations of PNA-ISH with other techniques like the flow cytometry (Kimura H et al., 2009; Baerlocher GM et al., 2006; Potter AJ and Wener NH, 2005), the fluorescence resonance energy transfer (Blanco AM et al., 2009) or the application of templated reactions (Pianowski Z et al., 2009) make this technique one of the most flexible and employed PNA application.

1.3.7.2 PCR Clamping

Individual genetic variants, are often defined by nucleotide sequence variations at single bases-level. The single nucleotide polymorphisms (SNPs) are among the genetic causes of the tremendous phenotypic diversity among the memebrs of a species. However, SNPs also play a relevant role in numerous human diseases and the identification of new SNPs and the development of effective diagnostic systems turned out to be crucial for the better understanding of the genetic bases of certain diseases. Polymorphisms are involved in single gene disease such as in emophilia and cystic fibrosis, have a preeminent role in polygenic diseases such as in diabetes and in several types of cancers and are implicated in individual differences in the immune system responses and in the aging processes. Several molecular methods have been developed to detect SNPs both for diagnostics and research purposes; the most common methods for SNPs analysis are DNA sequencing and restriction fragment-length polymorphism (RFLP). However, RFLP needs that polymorphisms occur in a restriction site in order to be detected and both methods require multiple procedural steps, are quite expensive and are often insensitive: a major problem in using somatic point mutations as markers for diseases, is that the analized samples usually contain the mutant allele in a

large excess of wild-tipe DNA. Moreover, the SNP of interest is sometimes embedded in special sequences particularly difficult to be detected (e.g. oligonucleotide repeat). Allele-specific PCR-based methods have been developed to detect trace amounts of point mutations, although the performances of this approach depend on the primer-template mismatch destabilization effect, which might result not enough effective.

PNA-mediated PCR clamping have been developed in order to overcome these limitations (Ørum H, 2000). The method takes advantage of the high termal stability and specificity of PNAs and is based on the competition for a common target site between a PCR primer and a PNA. If the PNA is complementary to the wild-type target sequence and the primer match the mutant target sequence, PNA binding will dominate over primer binding to the wild-type sequence and, since the PNA can not be extended by the Taq-polymerase, the amplification reaction will be blocked. When the mutant sequence is present, the PNA binding will result strongly destabilized, thus allowing the primer binding and the formation of the amplicons. The same technique has also been developed targeting sequences adjacent to one PCR primer or interfering with primer elongation. The PNA-mediated PCR clamping is very efficient and optimized assays can detect mutant templates in a ratio of 1:10,000 wild-type alleles (Luo JD et al., 2006). Numerous PCR clamping applications have been developed for mutations of genes involved in cancer such as K-ras (Chen CY et al., 2004; Taback B et al., 2004; Chiou CC et al., 2006; Luo JD et al., 2006; Gilje B et al., 2008; Beau-Faller M et al., 2009), or in rare diseases like the McCune-Albright syndrome (Bianco P et al., 2000; Lietman SA et al., 2005; Imanaka M et al., 2007) which is difficult to detect using conventional methods because due to mosaic mutations of the GNAS gene. PNA mediated PCR clamping have also been applied in the detection of pathernally inherited fetal point mutations for beta-thalassemia (Li Y et al., 2005), in detection of mitochondrial DNA mutations (Murdock DG et al., 2000) in combination with allele-specific PCR (Urata M et al., 2004), RFLP (Munakata K et al., 2005) and real time PCR (Thèves C et al., 2006). This technique has been extensively employed in virology for the detection of the emergence of lamivudine-resistant hepatitis B virus (HBV) (Kirishima T et al., 2002; Ogata N et al., 2003; Ohishi W and Chayama W, 2003; Ohishi W et al., 2004; Matsuda M et al., 2004). Tyrosine-methionine-aspartate-aspartate (YMDD) mutants can appear during prolonged treatment with lamivudine in chronic hepatitis B patients and effective detection of such mutations before or early is important to optimize the antiviral treatment.

Other applications of PCR clamping have been developed for food safety, for the detection of genetically modified organisms (GMO) (Peano C et al., 2005), for medical microbiology

(Iwamoto T and Sonobe T, 2004) and for the study of the diversity in complex microbial environments (von Wintzingerode et al., 2000). A variety of modified PNA-mediated PCR clamping has also been proposed for specific applications: assays involving PNA and LNA have been used to study mutations in the epidermal growth factor receptor (Nagai Y et al., 2005; Sutani A et al., 2006; Tanaka T et al., 2007; Miyazawa H et al., 2008). PNAs have been used as PCR clamp and sensor probes at the same time binding a fluorescein tag to the PNA probe which undergoes fluorescence resonance energy transfer with the adjacent fluorophore of an anchor probe when both are annealed to the template DNA (Luo JD et al., 2006; Chiou CC et al., 2006). PNA clamping has been involved in the development of new technologies like the Fluorescent Amplicon Generation (FLAG) assay that utilizes the exceptionally thermostable endonuclease PspGI for real-time signal generation by cleavage of quenched fluorophores from the 5'-end of the PCR products and PNAs, for selecting mutations over wild-type sequences (Amicarelli G et al., 2007).

1.3.7.3 PNAs in isothermal assays

Isothermal based amplification assays, such as Nucleic Acid Sequence-Based Amplification (NASBA) (Deiman B et al., 2002), Loop-Mediated Isothermal Amplification (LAMP) (Parida M et al., 2008, Mori Y and Notomi T, 2009) or Rolling Circle Amplification (RCA) (Zhao W et al., 2008) gained popularity in the last decade as effective alternatives to the PCR-based methods. These methods are commonly based on combinations of different enzyme: NASBA for example, is based on the mix of a reverse transcriptase (RT), an RNase H and a T7 DNA dependent RNA polymerase (T7 DdRp). The main advantages of the isothermal based assays are the absence of requirements for thermal cycling procedures and dedicated equipments, high amplification rates under optimum conditions up to 10^{12}-fold amplification in 90 minutes (Lau LT et al., 2006) and low carry-over contamination risks. Isothermal-based methods have been shown to be particularly suitable in clinical and environmental applications, for specific tasks such as viral RNA genome detection or bacterial viability evaluation (Keer JT and Birch L, 2003; Weile J and Knabbe C, 2009) or as signal enhancement strategies in specific assays. An interesting approach involving PNAs in a rolling circle amplification (RCA) process combined with an allosteric RNA-cleaving DNAzyme and a duplex binding dye has been recently reported: the assay relies on the allosteric DNAzyme cleavage in the presence of its substrate. This event generates a DNA primer to initiate RCA with the subsequent PNA annealing to the resulting long ssDNA. The duplex formation is than detected with the DiSC2(5) dye that binds the PNA/DNA duplex,

enabling naked eye detection (Ali MM and Li Y, 2009). Moreover, PNAs have been used as openers combined to RCA detection for analysis of short DNA sequences on the background of or within genomic DNA under non-denaturing conditions: double stranded DNA is locally opened by a pair of PNA openers thus allowing the RCA reaction (Smolina IV et al., 2008; Smolina I et al., 2007; Kuhn H et al., 2002b). Interestingly, examples of PNAs as probes or clamping elements in isothermal assays have not been yet extensively investigated.

1.3.7.4 PNA applications using microarray technologies

The increasing knowledge in the '-oms' domains (genome, proteome, transcriptome, metabolome, ecc) and the consequent needs for parellel processing of increasing amounts of informations have been the major driving force for the tremendous developments in the last decade of microarray technologies. Microarray assays originate from solid phase techniques used for decades such as DNA/RNA dot blot assays and ELISA (Dufva M, 2009). Initially developed as macroarrays made by membrane sheets spotted with cDNA (1-10,000 genes) used for comparative hybridization of RNA species (Peeters JK and Van der Spek P, 2005), this approach evolved in microarray thanks to the fabrication technologies advancement. The core principle behind microarrays is the possibility to immobilize many different probes onto a solid support resulting in a tremendous parallel analysis capacity; this approach allows to study the interactions of a whole population of molecules at the same time. A typical case of DNA/RNA microarray experiment usually involves a sample preparation phase by which nucleic acids are extracted and purified from cells or organisms, undergo an amplification process resulting in a target sequence enrichment and are labelled with detectable molecules such as fluorophores. The samples are then hybridized with the microarray probes under stringent conditions and the aspecific interactions are removed by washing procedures of the hybridized slide. The results are acquired with a high precision scanner (usually by means of fuorescence reading) and the data are processed with opportune softwares. However, several workflow variants have been developed and commonly used depending on the analytical purposes. Nowadays, numerous methods to bind oligonucleotides, PCR products, artificial chromosomes, peptides, proteins and other small molecules have been developed and several solid supports such as polymeric materials, glass and silicon along with numerous immobilization reactions have been evaluated for probe attachment to surfaces (Todt S and Blohm DH, 2009).

Microarrays are employed in a wide range of applications ranging from foundamental research purposes that use microarray for comparative genomic hybridization (CGH) and

analysis of gene expression to diagnostics of biomarkers (genotyping and determination of disease-relevant genes or disease-causative agents, mutation analysis, multiple single nucleotide polymorphisms (multi-SNPs) screening), genetic disorders (chromosomal abnormalities and mutations analysis, SNPs detection, global determination of post-transcriptional modifications including methylation, acetylation and alternative splicing) and infectious diseases (bacteria, virus and fungi detection) (Yoo SM et al., 2009).

Nowadays, a wide range of commercial microarray slides and dedicated instrumentations are available on the markets as ready-to-use devices and numerous patents and proprietary technologies have been developed (Kimmel A and Oliver B, 2006; Yoo SM et al., 2009; Duva M, 2009).

Microarray technologies and methods involving PNAs have also been proposed, in order to improve DNA detection capacity or meet the demand for optimization of specific applications.

Employment of PNAs instead of standard DNA probes present some advantages that might be useful to improve some microarray technical aspects: i) PNA probes improve sensitivity and selectivity of binding; ii) the neutral backbone of PNAs enables to expand the range of methods for the detection of the hybridized nucleic acids. Detection systems based on water-soluble cationic conjugated polymers are briliant examples of these innovative approaches: these methods take advantage of the net increase in negative charge that occurs when DNA or RNA molecules bind to a PNA probe allowing the local interaction with an oppositely charged cationic canjugated polymer. The resulting fluorescence emission can be then detected by conventional microarray reader (Liu B and Bazan GC, 2004; Raymond FR et al., 2005); iii) PNAs enable to expand the range of the operative conditions for nucleic acids hybridization: the use of PNA probes under low-salt concentration favours the DNA-PNA duplex over DNA-DNA duplex and promote the destabilization of intramolecular DNA structures that might reduce the accessibility to the targeted sequences and impair the hybridization to the probes; iv) PNA-based devices were shown to have better stability to some regeneration process and longer shelf-lives (Kröger et al., 2002). Recently a PNA microarray has been shown to be stable at room temperature storage for more than a year (Choi JJ et al., 2009). The PNA microarrays can be constructed using conventional automated robotic systems and certain types of custom PNA microarrays can also be designed and purchased from specialized companies (Panagene website).

Several microarray applications involving PNAs as binding probes have been shown to be effective altenatives to DNA-based detection systems in food safety, infectious desease

diagnostics and SNPs analysis: PNA based microarrays have been successfully employed for genetically modified organisms (GMOs) detection (Germini A et al., 2004b; Germini et al., 2005) and for the identification of hidden allergens in foodstuffs (Rossi S et al., 2006). Recently, the same technology has also been used for the detection and the simultaneous genotyping of 32 types of human papilloma virus (HPV) showing excellent specificity and sensitivity (Choi JJ et al., 2009). Moreover, a PNA zip-code microarray, combined with a multiplex single base extension (SBE) reaction, has been employed to detect mutations in the exon 2 of the hepatocyte nuclear factor-1a (HNF-1a) (Song JY et al., 2005) and a similar zip-code based strategy has been recently coupled with a single-strand specific (SSS) nuclease to genotype selected Korean-specific BRCA mutation sites. This method relies on the mismatched cleavage activity of the SSS nuclease for the SNP recognition and use the PNA tag for sorting the reaction products (Mun HY et al., 2009).

PNAs have also been used as tags, encoding a defined molecule by a specific sequence. Applications of PNA-tagged small-molecule libraries have been successfully applied to functional proteome profiling (Winssinger N et al., 2001; 2002). After a reaction in homogeneus solution, the tagged molecules are then separed and sorted using an array of probes complementary to the PNA tag sequences. A series of PNA-conjugated compounds designed to inhibit cathepsins were incubated with cathepsin C, allowed to flow through a size exclusion column to remove the library molecule that had not interacted with a protein and hybridized to a GenFlex tag array. This approach allowed the identification of specific cathepsin inhibitors, the rapid diagnostic identification of specific cathepsin activity in biological samples, and the identification of novel proteases. The methods have been further investigated with crude cell lysates enabling the detection of caspase activation upon induction of apoptosis, the characterization of the activated caspase, and the inhibition of the caspase-executed apoptotic phenotype using the small molecule inhibitor identified in the microarray-based profile (Winssinger N et al., 2002). Using a combinatorial approach, a 10,000 member peptide nucleic acid (PNA) encoded peptide library was treated with the Abelson tyrosine kinase (Abl) and decoded using a DNA microarray in order to identify the peptide sequences phosphorylated by Abl (Pouchain D et al., 2007). In this approach a lysine was used as branch molecule and PEG molecules as spacers. Similar approaches for screening of 10,000 protease substrates (Díaz-Mochón JJ et al., 2006) and smaller combinatorial libraries containing substrates for proteases or for the kinase Abl (Díaz-Mochón JJ et al., 2005) have also been reported.

Modified PNA probes designed in order to address the needs for higher sensitivity and specifity have been investigated in microarray formats. A class of peptide nucleic acids bearing a trans-cyclopentane constraint within the PNA backbone (tcypPNA) which has been shown to possess improved binding affinity and sequence specificity for their complementary DNA targets have been spotted onto amine-active slides and used in a nanoparticles scanometric DNA detection assay (Pokorski JK et al., 2005). Other modified PNAs containing one chiral monomer bearing two arginine-derived side chains have been proposed in a microarray format for highly specific SNPs recognition (Calabretta A et al., 2009).

1.3.7.5 Applications of PNAs in biosensors

The integration of PNAs in biosensor-based approaches is one of the most dynamic field of research and the continuous development of new PNA based methods and technologies is tremendously expanding the spectra of the versatile tools currently available for research and diagnostics. A wide spectra of well-established molecular biology-based methods and technological platforms are currently available for research or diagnostic purposes; however, the majority of these approaches require equipped laboratories and trained personnel. The growing connections between biology, chemistry and engineering related fields, allowed the design of new biosensors. Some general advantages can be envisaged for these methods: i) improved time-to-results and possibility of real time results acquisition; ii) (often) no needs for target labelling; iii) high miniaturization and automation potential (essential for high throughput or point-of-care devices); iv) better performances in terms of sensitivity and selectivity; v) possibility for direct nucleic acid detection (in some cases) without needs for nucleic acids amplification steps.

Several reasons explain the frequent employment of PNAs in this fast-growing research field: most of the biosensors are developed for specific purposes such as SNP detection or identification of traces amount of DNA/RNA specific sequences in a background of not-related nucleic acids and proteins. These methods can, therefore, take advantage of the higher selectivity and the enhanced binding affinity of PNAs compared to conventional DNA probes, thus improving the overall performances. Moreover, since the majority of the biosensor-based approaches are based on the detection of electrical perturbation signals or variations in optical signals, which often involve interactions with other charged molecules employed as signal enhancers or as substrates in signal-generating reactions, the neutral backbone of PNAs play an important role to avoid charge repulsion effects.

Systems based on surface plasmon resonance (SPR) technology are among the most developed biosensor involving PNA probes. This technique, exploits the surface plasmon (SP) electron density waves originated at the interface of a metal (e.g. gold) and a dielectric (e.g. glass) and monitors the changes in the refractive index derived from an analyte binding to a metal-coated surface of a prism (Dover JE et al., 2009). SPR enables real time monitoring of the hybridization events. Extensively used as effective method to carachterize PNA/DNA interactions (Jensen KK et al., 1997; Kambhampati D et al., 2001; Park H et al., 2006; 2007), SPR became soon a platform for diagnostic purposes. Exemples are the detection of *E. coli* O157:H7 from stool (Kai E et al., 2000) and the detection of toxic chemicals based on quantitative estimations of the induced P450 mRNAs in *Saccharomyces cerevisiae* detected directly or after NASBA amplification, to improve the sensitivity (Oyama M et al., 2000). Chemically modified PNA probes have also been employed in SPR analysis: chiral PNAs, obtained inserting three chiral monomers based on D-lysine, have been used to enhance mismatch recognition for the detection of the W1282X point mutation of the cystic fibrosis gene (Corradini R et al., 2004). Recently, pyrrolidinyl PNAs (HS-PNAs) bearing D-prolyl-2-aminocyclopentanecarboxylic acid (ACPC) backbones have been investigated using SPR (Ananthanawat C et al., 2009). Several signal amplification strategies such as DNA-templated polyaniline deposition (Su X et al., 2008), DNA-carrying hydrogel microspheres (Sato Y et al., 2003), ferrocene-streptavidin (Fc-Stv) conjugation for SPR and electrochemical detection (Liu J et al., 2006) or SPR improvements such as nanoparticle-enhanced SPR imaging (D'Agata R et al., 2008) have been investigated.

PNA probes have also been used for nanotubes and nanowires derivatization. Several application involving silicon nanowires (Gao Z et al., 2007; Zhang GJ et al., 2008a, 2008b) have been proposed. Recently, chip-based sensor arrays based on nanoelectromechanical resonators made of silicon and rhodium nanowires have been developed (Li M et al., 2008). A silicon nanowires-base device has been recently used for micro RNA (miRNA) detection from eucariotic cells (Zhang JG et al., 2009) and an electrode platform consisting of three-dimensional gold nanowires has been employed to directly detects specific RNA sequences (cancer biomarkers) in cellular and clinical samples without any sample labeling or PCR amplification (Fang Z and Kelley SO, 2009). Moreover, numerous PNA-based electrochemical detection methods employing a wide range of detection strategies have been developed: threading intercalators (Gao Z and Tansil N, 2009), electroactive polymers (Reisberg S et al., 2008), local formation of silver nanoparticles (Kong J et al., 2008), enzyme catalized reactions (Won BY et al., 2008), conducting polymer nanowires (Fan Y et al., 2007)

are among the approaches developed for signals enhancement and detection. Immobilization-free detection strategies involving a solution-phase hybridization step have also been suggested as effective alternatives (Luo X and Hsing IM, 2009; Luo X et al., 2008).
Other devices like leaky surface acoustic wave (LSAW) bis-PNA (bis-PNA) biosensor (Wang Y et al., 2009) used for directly detect HPV genomic DNA without polymerase chain reaction (PCR) amplification, quartz crystal microbalances (QCM) used for the real-time monitoring of hepatitis B virus (HBV) genomic DNA hybridization (Yao C et al., 2008) and microfluidic devices for bacterial detection using PNA beacons (Xi C et al, 2005) or DNA mapping using fluorescent bisPNA (Chan EY et al., 2004) are interesting examples of biosensor-based devices involving PNAs.

1.3.8 Applications of PNA-based systems in food safety and authenticity

PNA-based methods have been applied extensively as tools for basic research and molecular medicine and biology. Moreover, the peculiar properties of PNAs have led to the development of numerous application for diagnostics, where PNAs have been used mainly as probes in both clinical and environmental settings. Beside these applications, PNAs have also been successfully employed for food safety- and authenticity-oriented applications.
Microarrays bearing PNAs oligomers as specific probes have been developed for detection of genetically modified organisms (GMOs). A PNA microarray for the detection of Roundup Ready soybeans in food have been prepared and applied to the analysis of a sample of certified transgenic soybean flour (Germini et al., 2004). In this work, DNA extracted from reference material was amplified using Cy3- and Cy5-labeled primers, and the products obtained were hybridized on the microarray studing two different protocols based on the hybridization with dsDNA or ssDNA obtained by enzymatic digestion. Moreover the hybridization efficiency of PNA probes with different length have been evaluated for both the ssDNA- and the dsDNA-based hybridization protocol: the best results were obtained using a 15-mer PNA probe in combination with the ssPCR product derived from enzymatic digestion; however it has been demonstrated the possibility to hybridize the amplified dsDNA directly onto the microarray, at least for one of the designed probe.
The same strategy combined with a multiplex PCR amplification system, have been succesfully applied for the GMOs identification in flour samples containing a mixture of standards at known concentrations of transgenic Roundup Ready soybean and Bt11, Bt176, Mon810, and GA21 maize (Germini et al., 2005). In this work, the PNA-microarray design have been further investigated in terms of best PNA probes length and spacing from the slide

surface. A similar platform combined with a duplex PCR have been used to simultaneously detect the presence of hidden allergens (hazelnut and peanut) in raw materials and commercial foodstuffs (Rossi S et al., 2006).

PNAs have also been used as probes to specifically detect GMOs (Roundup Ready soybean or Bt-176 maize) in an anion-exchange HPLC assay: ssDNA PCR products obtained by asymmetric PCR or enzymatic digestion have been hybridized with specific PNA probes enabling thus the detection of the corresponding PNA:DNA peak with significantly different retention time (Rossi S et al., 2007).

GMOs content in food has also been semiqualtitatively evaluated using a PNA-based PCR clamping assay (Peano C et al., 2005). In this work, an optimization of PCR clamping by PNA has been performed by testing five PNAs with different length.

A method based on a PNA probe combined with the 3,3'-diethylthiadicarbocyanine dye [DiSC(2)(5)] has been developed to detect a tract of the peanut Ara h 2 gene (Sforza et al., 2005). The DiSC(2)(5) dye aggregates onto the PNA-DNA duplex giving rise to a characteristic emission signal. Exploiting the right-handed helical conformation of the PNA-DNA duplex the aggregation of the dye to the duplex can be monitored using circular dichroism (CD) spectroscopy. The method has been applied to the identification and quantitation of DNA extracted and amplified by PCR from peanuts and from peanut-containing foods, allowing for a very sensitive detection at a very low level (few pmol).

Moreover, a PNA microarray for the recognition of different olive tree cultivars is under development (Rossi S, 2007) with the aim to provide a suitable device for frauds detection in the olive oil production. Further improvements using PNAs probe combined with ultra-sensitive techniques allow to detect specific nucleic acid sequences without any amplification procedure. A PNA probe has been used for directly detect extracted nucleic acids from a GMO standard material (flour samples) using the surface plasmon resonance imaging (SPRi) technique.

The current demand for sensitive and easy methods for food diagnostics make of PNAs invaluable tools for the development of advanced diagnostic methods.

1.3.9 Other PNA applications in biotechnology and molecular biology

The outstanding characteristics and the versatility of PNAs have led to the develoment of a myriad of applications spanning the needs of numerous disciplines. Apart from the applications described in detail in the previous paragraphs, other notable PNA-based approaches will be briefly described here:

PNA-based templated reactions.

Templated reactions are based on the principle that a suitable template binds the reactants and aligns the reactive groups allowing conversions to occur faster than in the absence of the template, simirarly to the catalysed reactions. DNA-templated reactions have been investigated for programmed assembly of DNA-based nanomaterials, the construction of amplifiable small-molecule libraries, the specific detectione of the nucleic acids and for sequence-specific release of drugs (Grossmann TN et al., 2008). PNAs have been used in native chemical reactions in which a PNA-glycine thioester reacts with an iso-cystein-PNA leading to a ligation and with a subsequent extension of the main-chain; this event increases the flexibility of the ligation intermediate and plays a destabilizing effect on the product–template duplex enabling a reaction turnover. This approach combined with a FRET system has been used for mismatch recognition (Dose C et al., 2006). PNAs have also been involved in DNA-catalysed Staudinger reactions: a low-fluorescent azido-coumarin, and a triphenylphosphine-modified PNA probe in the presence of a catalytic amount of DNA template induced the reduction of the azide function, releasing nitrogen and yielding a highly fluorescent coumarin derivative. The reaction has been demonstrated with a single nucleotide resolution (Pianowski ZL and Winssinger N, 2007). Recently the reduction of azidorhodamine PNA-probes has been showed to be an effective tool for imaging of mRNA in living cells (Pianowski Z et al., 2009).

PNAs as DNA cutters and artificial restriction enzymes.

Site-specific digestion of DNA have been traditionally carried out using restriction enzymes. However, this approach presents some limitations related to potential difficulties in finding the appropriate restriction enzyme for the desired manipulation. Furthermore, naturally occurring restriction enzymes usually recognize short sequences (4-6 bp) and the number of restriction sites might result too high using large genomic DNA molecules. PNAs have been proposed as effective tools to cut large DNA molecules site selectively in order to allow their precise manipulation. Site specific digestion of dsDNA have been accomplished by means of PNAs in the presence of the single strand specific nuclease S1 (Demidov V et al., 1993) and the sequence selective hydrolysis of linear ssDNA has been reported using PNA conjugated with Zr(IV)-complexes (Zelder FH et al., 2003). Recently, the artificial restriction DNA cutter (ARCUT) method to cut double-stranded DNA at designated sites has been proposed; the ARCUT system is composed of a CeIV–EDTA complex (a catalyst for DNA hydrolysis) and a pair of pcPNA for sequence recognition (Komiyama M et al., 2008). The partially overlapping pcPNAs open the ds DNA and provide single-stranded portions at the binding

site that can be cutted by the CeIV–EDTA complex which hydrolyzes only DNA in the single-stranded state. The potency of the ARCUT system has been showed by performing several DNA manipulations using artificial vectors and genomic DNA (Katada H and Komiyama M, 2009; Ito K et al., 2009).

PNA-based bioseparations.

In addition to several standard methods of DNA purification such as anion-exchange chromatography and density-gradient techniques, PNA-based affinity separations have been proposed as effective strategies for sequence specific nucleic acid separation. The hybridization of PNA to DNA or RNA has been exploited in analytical applications based on electrophoresis. The PNA binding changes the electrophoretic mobility, affecting the charge: size ratio; under optimized conditions even single mismatch-resolution separations can be performed. This principle has been applied to diagnostics; for example, a chiral peptide nucleic acid probe has been employed to detect the R553X DNA single point mutation related to cystic fibrosis by capillary electrophoresis (Tedeschi T et al., 2005).

The hybridization of PNAs to nucleic acids has also been investigated for several large-scale purification purposes, ranging from purification of plasmids or genomic DNA to RNA and RNA-protein complexes. Purifications based on PNA hybridizations in solution, followed by the capture of PNA-DNA(RNA) complexes by interaction of beads or surfaces have also been demonstrated. A (His)6-PNA chimera has been used to separate sequences differing by only a single nucleotide and large RNAs. Purification of an oligonucleotide in which the target sequence forms part of an intramolecular stem/loop structure has been performed using low-salt concentrations that destabilize native nucleic acid structures (Orum H et al., 1995). Biotinylated PNAs has been used to hybridize human genomic DNA in solution forming a high-affinity triplex with A7 sequence motifs in the target DNA. The complex was then captured onto paramagnetic streptavidin-coated particles, which were subsequently used for PCR reactions. The method has been shown to be effective in removing inhibitors of the PCR from bloods (Seeger C et al., 1997). Recently, an application of PNA as an affinity capture reagent has been proposed for the study of RNA-protein complexes (RNPs) in cells (Zeng F et al., 2006; Zielinski J et al., 2006). The peptide nucleic acid (PNA)-assisted identification of RNA-binding proteins (RBPs) (PAIR) technology relies on a cell membrane-penetrating peptide (CPP)-linked PNA oligomer that complements a target mRNA sequence and anneals to it in the living cell. PNA is then covalently coupled to the mRNA-RBP complexes through an ultraviolet (UV) cross-linking step. The resulting PNA-RNA-RBP complex can be isolated using sense oligonucleotide magnetic beads, and the RBPs can then be identified by mass

spectrometry (MS). This method has been developed in order to overcome the current limitations in the identification of RBPs that specifically associate with selected RNAs, particularly in live cells, and it has been used to study protein association to the ankylosis mRNA in cortical neurons and pharmacological modulation of these specific protein–RNA associations (Zielinski J et al., 2006).

1.3.10 PNA applications in medicine

The ability of PNAs to bind sequence-selectively nucleic acids, even in double strand forms, opened an interesting scenario in medicinal chemistry and biotechnology raising interest for PNA applications as antisense and antigene tools for gene expression regulation. PNAs soon become one of the most preferred tool to accomplish these tasks: the higher affinity and sequence selectivity compared to standard DNA, the biochemical stability to nuclease and protease and the wide derivatization possibilities make PNAs suitable tools for *in vitro* and *in vivo* appications involving gene expression modulation at both DNA and RNA levels.

The classic DNA-based antisense strategies rely on the binding of an oligonucleotide to a complementary sequence of a targeted mRNA with the consequent block of translational events. The antisense effect basically occurs involving at least one of the following mechanisms: i) block of the ribosome complex formation; ii) premature interruption of the transcription due to steric block in a coding region; iii) degradation of the antisense:mRNA complex by activation of specific RNases. Since PNAs are stable in presence of cellular nucleases and proteases, the PNA-mediated antisense strategies rely on the first two mechanisms; however it has been shown that targeting a coding mRNA region might result in an unefficient blocking of translation, whereas targeting sequences located in the 5'-untranslated region seems to provide a stronger antisense effect (Doyle DF et al., 2001). PNAs as antisense agents have been investigated for: i) viruses infections such as in the inhibition of the Japanese encephalitis virus replication (Yoo JS et al., 2009); Tat-mediated transactivation of the HIV-1 long terminal repeat (LTR) sequences has been efficiently inhibited by PNAs *via* binding to the targeted site on TAR RNA (Kaushik N et al., 2002). One of the investigated PNA exhibited a 99% reduction of HIV-1 production showing the role of PNAs as potential anti-HIV agents (Pandey VN et al., 2009); ii) allele-specific silencing: a PNA has been used to silence mutant huntingtin and ataxin-3 genes by targeting expanded CAG repeats in mRNAs (Hu J et al.,2009); this approach suggest new applications for antisense oligomers that discriminate between wild-type and mutant genes on the basis of repeat length that may offer new options for developing treatments; iii) target bacterial genes.

For example, PNA-based antisense technology has been effectively empolyed to suppress the function of the CmeABC multidrug efflux transporter, opening possibilities to control antibiotic-resistant *Campylobacter* (Jeon B and Zhang Q, 2009); iv) disrupt genetic mechanisms involved cancer by means of PNA-based tumor-specific agents; a PNA has been used for the selective inhibition of NMYC in neuroblastoma cells targeting a unique sequence in the terminus of the 5'-UTR of N-myc (Pession A et al., 2004).

Since many genetic deseases originate from wrong events during the pre-mRNA maturation steps, RNA-targeted PNAs are also employed to regulate mechanisms at a non-messenger level. PNAs have been used to target pre-mRNA splicing sites in order to shift the protein expression pattern in favour of the desired form (Sazani P et al., 2002) or to force exon skipping events. The restoration of the correct reading frame with the subsequent expression of functional dystrophin protein is an effective example of PNA-mediated exon skipping that may play an important role in the antisense therapy of the Duchenne muscular dystrophy (Yin H et al., 2008).

PNAs have also been investigated as compounds for developing gene therapeutic drugs. This principle has been applied targeting functional and accessible sites in ribosomal RNA in order to shut down the whole protein syntesis machinery (Good L and Nielsen PE, 1998) opening interesting possibilities in the sequence-specific antibiotic activity of PNAs.

PNAs play an important role in targeting types of RNAs different from pre- and mature mRNA.

PNAs targeting the RNA component of telomerase have been used to block the telomerase activity *in vitro* and in cell cultures (Herbert B et al., 1999; Hamilton SE et al.,1999): since a correlation between the telomerase activity and human tumors has been envisaged, telomerase inhibitors represent a class of chemotherapeutic agents and PNAs can play and important role in this field, acting at a sequence-specific level.

PNAs have also been investigated for microRNA knockdown (Fabani MM and Gait MJ), showing their potential for future therapeutic applications as well as for studying microRNA functions. PNA-based antisense strategies have also been used for research purposes such as the study of expression pathways and gene interactions (Faridani OR et al., 2006).

The ability of PNAs to bind dsDNA in a sequence-selective manner, raised interests in using PNAs as anti-gene agents. A PNA-based anti-gene strategy has been used for the selective inhibition of MYCN transcription in neuroblastoma cells, targeting a unique sequence in the antisense DNA strand of exon 2 of MYCN (Tonelli R et al., 2005). The suppression of HIV-1

replication in chronically infected lymphocytes and macrophages and efficiently prevention of mitogen-induced HIV-1 reactivation in lymphocytes has been achieved by an anti-gene PNA (Pesce CD et al., 2005); this approach was investigated in order to overcome the limitations of the highly active antiretroviral therapy (HAART) which is unlikely to affect reservoirs of HIV in latently infected cells.

Antisense and anti-gene PNAs have been shown to be powerful tools for therapeutic applications; however, before the extensive employment of these techniques in medical settings, major improvement in cell delivery, binding kinetics, side- and toxic- effects evaluations must be achieved.

1.4 REFERENCES

Albinana-Gimenez N, Clemente-Casares P, Calgua B, Huguet JM, Courtois S, Girones R. (2009) Comparison of methods for concentrating human adenoviruses, polyomavirus JC and noroviruses in source waters and drinking water using quantitative PCR. J Virol Methods; 158(1-2):104-9.

Alexander BD, Ashley ED, Reller LB, Reed SD. (2006) Cost savings with implementation of PNA FISH testing for identification of Candida albicans in blood cultures. Diagn Microbiol Infect Dis;54(4):277-82.

Ali MM, Li Y. (2009) Colorimetric sensing by using allosteric-DNAzyme-coupled rolling circle amplification and a peptide nucleic acid-organic dye probe. Angew Chem Int Ed Engl.;48(19):3512-5.

Almeida C, Azevedo NF, Iversen C, Fanning S, Keevil CW, Vieira MJ. (2009) Development and application of a novel peptide nucleic acid probe for the specific detection of Cronobacter genomospecies (Enterobacter sakazakii) in powdered infant formula. Appl Environ Microbiol;75(9):2925-30.

Amicarelli G, Shehi E, Makrigiorgos GM, Adlerstein D. (2007) FLAG assay as a novel method for real-time signal generation during PCR: application to detection and genotyping of KRAS codon 12 mutations. Nucleic Acids Res.;35(19):e131.

Ananthanawat C, Vilaivan T, Mekboonsonglarp W, Hoven VP. (2009) Thiolated pyrrolidinyl peptide nucleic acids for the detection of DNA hybridization using surface plasmon resonance. Biosens Bioelectron. 15;24(12):3544-9.

Ando T, Noel JS, Fankhauser RL. (2000) Genetic classification of "Norwalk-like viruses.. J Infect Dis;181 Suppl 2:S336-48.

Ando T, Jin Q, Gentsch JR, Monroe SS, Noel JS, Dowell SF, Cicirello HG, Kohn MA, Glass RI. (1995) Epidemiologic applications of novel molecular methods to detect and differentiate small round structured viruses (Norwalk-like viruses). J Med Virol;47(2):145-52.

Antonishyn NA, Crozier NA, McDonald RR, Levett PN, Horsman GB. (2006) Rapid detection of Norovirus based on an automated extraction protocol and a real-time multiplexed single-step RT-PCR. J Clin Virol;37(3):156-61.

Asahina AY, Lu Y, Wu C, Fujioka RS, Loh PC. (2009) Potential biosentinels of human waste in marine coastal waters: bioaccumulation of human noroviruses and enteroviruses from sewage-polluted waters by indigenous mollusks. J Virol Methods;158(1-2):46-50.

Atmar RL, Estes MK. (2001) Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses. Clin Microbiol Rev. Jan;14(1):15-37.

Aw TG, Gin KY, Ean Oon LL, Chen EX, Woo CH. (2009) Prevalence and genotypes of human noroviruses in tropical urban surface waters and clinical samples in Singapore. Appl Environ Microbiol;75(15):4984-92.

Ayodeji M, Kulka M, Jackson SA, Patel I, Mammel M, Cebula TA, Goswami BB. (2009) A microarray based approach for the identification of common foodborne viruses. Open Virol J.19;3:7-20.

Baerlocher GM, Vulto I, de Jong G, Lansdorp PM. (2006) Flow cytometry and FISH to measure the average length of telomeres (flow FISH). Nat Protoc;1(5):2365-76.

Baert L, Uyttendaele M, Stals A, VAN Coillie E, Dierick K, Debevere J, Botteldoorn N. (2009) Reported foodborne outbreaks due to noroviruses in Belgium: the link between food and patient investigations in an international context. Epidemiol Infect;137(3):316-25.
.

Baert L, Uyttendaele M, Vermeersch M, Van Coillie E, Debevere J. (2008a) Survival and transfer of murine norovirus 1, a surrogate for human noroviruses, during the production process of deep-frozen onions and spinach. J Food Prot;71(8):1590-7.

Baert L, Uyttendaele M, Van Coillie E, Debevere J. (2008b) The reduction of murine norovirus 1, B. fragilis HSP40 infecting phage B40-8 and E. coli after a mild thermal pasteurization process of raspberry puree. Food Microbiol;25(7):871-4

Baert L, Vandekinderen I, Devlieghere F, Van Coillie E, Debevere J, Uyttendaele M. (2008c) Inactivation of murine norovirus 1 and Bacteroides fragilis infecting phage B40-8 by the use of sodium hypochlorite and peroxyacetic acid as decontaminating agents for shredded iceberg lettuce. Commun Agric Appl Biol Sci.;73(1):97-101.

Baert L, Wobus CE, Van Coillie E, Thackray LB, Debevere J, Uyttendaele M. (2008d) Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. Appl Environ Microbiol;74(2):543-6.

Baert L, Uyttendaele M, Debevere J. (2008e) Evaluation of viral extraction methods on a broad range of Ready-To-Eat foods with conventional and real-time RT-PCR for Norovirus GII detection. Int J Food Microbiol 31;123(1-2):101-8.

Beau-Faller M, Legrain M, Voegeli AC, Guérin E, Lavaux T, Ruppert AM, Neuville A, Massard G, Wihlm JM, Quoix E, Oudet P, Gaub MP. (2009) Detection of K-Ras mutations in tumour samples of patients with non-small cell lung cancer using PNA-mediated PCR clamping. Br J Cancer;100(6):985-92.

Bethge L, Jarikote DV, Seitz O. (2008) New cyanine dyes as base surrogates in PNA: forced intercalation probes (FIT-probes) for homogeneous SNP detection. Bioorg Med Chem;16(1):114-25.

Betts L, Josey JA, Veal JM, Jordan SR. (1995) A nucleic acid triple helix formed by a peptide nucleic acid-DNA complex. Science;270(5243):1838-41

Beuret C. (2004) Simultaneous detection of enteric viruses by multiplex real-time RT-PCR. J Virol Methods;115(1):1-8.

Beuret C. (2003) A simple method for isolation of enteric viruses (noroviruses and enteroviruses) in water. J Virol Methods;107(1):1-8.

Beuret C, Kohler D, Baumgartner A, Lüthi TM. (2002) Norwalk-like virus sequences in mineral waters: one-year monitoring of three brands. Appl Environ Microbiol;68(4):1925-31.

Bianco P, Riminucci M, Majolagbe A, Kuznetsov SA, Collins MT, Mankani MH, Corsi A, Bone HG, Wientroub S, Spiegel AM, Fisher LW, Robey PG. (2000) Mutations of the GNAS1 gene, stromal cell dysfunction, and osteomalacic changes in non-McCune-Albright fibrous dysplasia of bone. J Bone Miner Res;15(1):120-8.

Bishop JA, Chase N, Magill SS, Kurtzman CP, Fiandaca MJ, Merz WG. (2008) Candida bracarensis detected among isolates of Candida glabrata by peptide nucleic acid fluorescence in situ hybridization: susceptibility data and documentation of presumed infection. J Clin Microbiol;46(2):443-6.

Blanco AM, Rausell L, Aguado B, Perez-Alonso M, Artero R. (2009) A FRET-based assay for characterization of alternative splicing events using peptide nucleic acid fluorescence in situ hybridization. Nucleic Acids Res;37(17):e116.

Boei JJ, Vermeulen S, Natarajan AT. (2000) Analysis of radiation-induced chromosomal aberrations using telomeric and centromeric PNA probes. Int J Radiat Biol;76(2):163-7.

Boei JJ, Vermeulen S, Fomina J, Natarajan AT. (1998) Detection of incomplete exchanges and interstitial fragments in X-irradiated human lymphocytes using a telomeric PNA probe. Int J Radiat Biol;73(6):599-603.

Bonvicini F, Mirasoli M, Gallinella G, Zerbini M, Musiani M, Roda A. (2007) PNA-based probe for quantitative chemiluminescent in situ hybridisation imaging of cellular parvovirus B19 replication kinetics. Analyst;132(6):519-23.

Bonvicini F, Filippone C, Manaresi E, Gentilomi GA, Zerbini M, Musiani M, Gallinella G. (2006) Peptide nucleic acid-based in situ hybridization assay for detection of parvovirus B19 nucleic acids. Clin Chem;52(6):973-8.

Bosch A, Guix S, Sano D, Pintó RM. (2008) New tools for the study and direct surveillance of viral pathogens in water. Curr Opin Biotechnol;19(3):295-301.

Boxman I, Dijkman R, Verhoef L, Maat A, van Dijk G, Vennema H, Koopmans M. Norovirus on swabs taken from hands illustrate route of transmission: a case study. J Food Prot. 2009 Aug;72(8):1753-5.

Boxman IL, Dijkman R, te Loeke NA, Hägele G, Tilburg JJ, Vennema H, Koopmans M (2009). Environmental swabs as a tool in norovirus outbreak investigation, including outbreaks on cruise ships. J Food Prot;72(1):111-9

Brinkman NE, Fout GS. (2009) Development and evaluation of a generic tag array to detect and genotype noroviruses in water. J Virol Methods;156(1-2):8-18.

Brown D., Gray J., MacDonald P., Green A., Morgan D., Christopher G., Glass R., Turcois R. (2002). Outbreak of acute gastroenteritis associated with Norwalk-like viruses among British military personnel--Afghanistan, May 2002. *MMWR Morb Mortal Wkly Rep* 51:477-9

Brown SC, Thomson SA, Veal JM, Davis DG. (1994) NMR solution structure of a peptide nucleic acid complexed with RNA. Science;265(5173):777-80

Bucardo F, Nordgren J, Carlsson B, Paniagua M, Lindgren PE, Espinoza F, Svensson L. (2008) Pediatric norovirus diarrhea in Nicaragua. J Clin Microbiol;46(8):2573-80.

Buchardt O, Egholm M, Nielsen PE, Berg, RH (1992). Peptide nucleic acids. In "PCT Int. Appl., Wo 92/20702," p. 192.

Butot S, Putallaz T, Amoroso R, Sánchez G. (2009) Inactivation of enteric viruses in minimally processed berries and herbs. Appl Environ Microbiol;75(12):4155-61.

Butot S, Putallaz T, Sánchez G. (2008) Effects of sanitation, freezing and frozen storage on enteric viruses in berries and herbs. Int J Food Microbiol;126(1-2):30-5.

Butot S, Putallaz T, Croquet C, Lamothe G, Meyer R, Joosten H, Sánchez G. (2007a) Attachment of enteric viruses to bottles. Appl Environ Microbiol;73(16):5104-10.

Butot S, Putallaz T, Sánchez G. (2007b) Procedure for rapid concentration and detection of enteric viruses from berries and vegetables. Appl Environ Microbiol;73(1):186-92.

Calabretta A, Tedeschi T, Di Cola G, Corradini R, Sforza S, Marchelli R (2009). Arginine-based PNA microarrays for APOE genotyping. Mol Biosyst. Nov;5(11):1323-30.

Cannon JL, Vinjé J (2008). Histo-blood group antigen assay for detecting noroviruses in water. Appl Environ Microbiol;74(21):6818-9.

Carter MJ (2005). Enterically infecting viruses: pathogenicity, transmission and significance for food and waterborne infection. J Appl Microbiol. ;98(6):1354-80.

Centers for Disease Control and Prevention (CDC), 2009. Surveillance for foodborne disease outbreaks - United States, 2006. MMWR Morb Mortal Wkly Rep. Jun 12;58(22):609-15.

CDC website: http://www.cdc.gov/

Chadwick PR, Beards G, Brown D, Caul EO, Cheesbrough J, Clarke I, Curry A, O'Brien S, Quigley K, Sellwood J, Westmoreland D (2000). Management of hospital outbreaks of gastro-enteritis due to small roundstructured viruses. J Hosp Infect;45(1):1-10.

Chan EY, Goncalves NM, Haeusler RA, Hatch AJ, Larson JW, Maletta AM, Yantz GR, Carstea ED, Fuchs M, Wong GG, Gullans SR, Gilmanshin R (2004). DNA mapping using microfluidic stretching and single-molecule detection of fluorescent site-specific tags. Genome Res;14(6):1137-46.

Chan MC, Wong YP, Leung WK (2007). Cell culture assay for human noroviruses. Emerg Infect Dis;13(7):1117; author reply 1117-8.

Chen C, Wu B, Wei T, Egholm M, Strauss WM (2000). Unique chromosome identification and sequence-specific structural analysis with short PNA oligomers. Mamm Genome;11(5):384-91.

Chen CY, Shiesh SC, Wu SJ (2004). Rapid detection of K-ras mutations in bile by peptide nucleic acid-mediated PCR clamping and melting curve analysis: comparison with restriction fragment length polymorphism analysis. Clin Chem;50(3):481-9.

Chen R, Neill JD, Noel JS, Hutson AM, Glass RI, Estes MK, Prasad BV (2004). Inter- and intragenus structural variations in caliciviruses and their functional implications. J Virol;78(12):6469-79.

Cheong S, Lee C, Choi WC, Lee CH, Kim SJ (2009a). Concentration method for the detection of enteric viruses from large volumes of foods. J Food Prot;72(9):2001-5.

Cheong S, Lee C, Song SW, Choi WC, Lee CH, Kim SJ (2009b). Enteric viruses in raw vegetables and groundwater used for irrigation in South Korea. Appl Environ Microbiol;75(24):7745-51.

Chiou CC, Luo JD, Chen TL (2006). Single-tube reaction using peptide nucleic acid as both PCR clamp and sensor probe for the detection of rare mutations. Nat Protoc.;1(6):2604-12.

Choi JJ, Kim C, Park H (2009). Peptide nucleic acid-based array for detecting and genotyping human papillomaviruses. J Clin Microbiol;47(6):1785-90.

Comelli HL, Rimstad E, Larsen S, Myrmel M (2008). Detection of norovirus genotype I.3b and II.4 in bioaccumulated blue mussels using different virus recovery methods. Int J Food Microbiol;127(1-2):53-9.

Corradini R, Feriotto G, Sforza S, Marchelli R, Gambari R (2004). Enhanced recognition of cystic fibrosis W1282X DNA point mutation by chiral peptide nucleic acid probes by a surface plasmon resonance biosensor. J Mol Recognit;17(1):76-84.

D'Agata R, Corradini R, Grasso G, Marchelli R, Spoto G (2008). Ultrasensitive detection of DNA by PNA and nanoparticle-enhanced surface plasmon resonance imaging. Chembiochem;9(13):2067-70.

da Silva AK, Le Saux JC, Parnaudeau S, Pommepuy M, Elimelech M, Le Guyader FS (2007). Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: different behaviors of genogroups I and II. Appl Environ Microbiol;73(24):7891-7.

Dall'Asta C, Galaverna G, Mangia M, Sforza S, Dossena A, Marchelli R (2009). Free and bound fumonisins in gluten-free food products. Mol Nutr Food Res;53(4):492-9.

de Bruin E, Duizer E, Vennema H, Koopmans MP (2006). Diagnosis of Norovirus outbreaks by commercial ELISA or RT-PCR. J Virol Methods;137(2):259-64.

de la Fuente MA, Juárez M (2005). Authenticity assessment of dairy products. Crit Rev Food Sci Nutr.;45(7-8):563-85.

de Pauw ES, Verwoerd NP, Duinkerken N, Willemze R, Raap AK, Fibbe WE, Tanke HJ (1998). Assessment of telomere length in hematopoietic interphase cells using in situ hybridization and digital fluorescence microscopy. Cytometry;32(3):163-9.

Deiman B, van Aarle P, Sillekens P (2002). Characteristics and applications of nucleic acid sequence-based amplification (NASBA). Mol Biotechnol;20(2):163-79.

Demidov V, Frank-Kamenetskii MD, Egholm M, Buchardt O, Nielsen PE (1993). Sequence selective double strand DNA cleavage by peptide nucleic acid (PNA) targeting using nuclease S1. Nucleic Acids Res;21(9):2103-7.

Demidov VV, Potaman VN, Frank-Kamenetskii MD, Egholm M, Buchard O, Sönnichsen SH, Nielsen PE (1994). Stability of peptide nucleic acids in human serum and cellular extracts. Biochem Pharmacol;48(6):1310-3.

Deng W, Lucas JN (1999). Combined FISH with pan-telomeric PNA and whole chromosome-specific DNA probes to detect complete and incomplete chromosomal exchanges in human lymphocytes. Int J Radiat Biol;75(9):1107-12.

Dimitriadis A, Bruggink LD, Marshall JA (2006). Evaluation of the Dako IDEIA norovirus EIA assay for detection of norovirus using faecal specimens from Australian gastroenteritis outbreaks. Pathology;38(2):157-65.

Donaldson EF, Lindesmith LC, Lobue AD, Baric RS (2008). Norovirus pathogenesis: mechanisms of persistence and immune evasion in human populations. Immunol Rev;225:190-211.

Dose C, Ficht S, Seitz O (2006). Reducing product inhibition in DNA-template-controlled ligation reactions. Angew Chem Int Ed Engl;45(32):5369-73.

Dover JE, Hwang GM, Mullen EH, Prorok BC, Suh SJ (2009). Recent advances in peptide probe-based biosensors for detection of infectious agents. J Microbiol Method;78(1):10-9.

Doyle DF, Braasch DA, Simmons CG, Janowski BA, Corey DR (2001). Inhibition of gene expression inside cells by peptide nucleic acids: effect of mRNA target sequence, mismatched bases, and PNA length. Biochemistry;40(1):53-64.

Dreier J, Störmer M, Mäde D, Burkhardt S, Kleesiek K (2006). Enhanced reverse transcription-PCR assay for detection of norovirus genogroup I. J Clin Microbiol;44(8):2714-20.

Drobniewski FA, More PG, Harris GS (2000). Differentiation of Mycobacterium tuberculosis complex and nontuberculous mycobacterial liquid cultures by using peptide nucleic acid-fluorescence In situ hybridization probes. J Clin Microbiol;38(1):444-7.

Dufva M (2009). Introduction to microarray technology. Methods Mol Biol.;529:1-22.

Díaz-Mochón JJ, Bialy L, Bradley M (2006). Dual colour, microarray-based, analysis of 10,000 protease substrates. Chem Commun;(38):3984-6.

Díaz-Mochón JJ, Bialy L, Keinicke L, Bradley M (2005). Combinatorial libraries - from solution to 2D microarrays. Chem Commun;(11):1384-6.

EFSA Journal (2009): The Community Summary Report on Food-borne Outbreaks in the European Union in 2007, *The EFSA Journal* (2009), 271.

Egholm M, Christensen L, Dueholm KL, Buchardt O, Coull J, Nielsen PE (1995). Efficient pH-independent sequence-specific DNA binding by pseudoisocytosine-containing bis-PNA. Nucleic Acids Res;23(2):217-22.

Eriksson M, Nielsen PE (1996). Solution structure of a peptide nucleic acid-DNA duplex. Nat Struct Biol;3(5):410-3.

Fabani MM, Gait MJ (2008). miR-122 targeting with LNA/2'-O-methyl oligonucleotide mixmers, peptide nucleic acids (PNA), and PNA-peptide conjugates. RNA;14(2):336-46.

Fan Y, Chen X, Trigg AD, Tung CH, Kong J, Gao Z (2007). Detection of MicroRNAs using target-guided formation of conducting polymer nanowires in nanogaps. J Am Chem Soc;129(17):5437-43.

Fang Z, Kelley SO (2009). Direct electrocatalytic mRNA detection using PNA-nanowire sensors. Anal Chem;81(2):612-7.

Fankhauser RL, Monroe SS, Noel JS, Humphrey CD, Bresee JS, Parashar UD, Ando T, Glass RI (2002). Epidemiologic and molecular trends of "Norwalk-like viruses" associated with outbreaks of gastroenteritis in the United States. J Infect Dis;186(1):1-7.

Faridani OR, Nikravesh A, Pandey DP, Gerdes K, Good L (2006). Competitive inhibition of natural antisense Sok-RNA interactions activates Hok-mediated cell killing in Escherichia coli. Nucleic Acids Res.;34(20):5915-22.

Flaqué MC, Bianchi MS, Bolzán AD (2006). A comparative analysis of bleomycin-induced incomplete chromosome elements in two mammalian cell lines using a telomeric PNA probe. Environ Mol Mutagen;47(9):674-81.

Forgacs E and Cserhati T (2006). Classification of chilli powders by high performance liquid chromatography-diode array detection . *Analytical Letters*, 39, 2775 – 2785.

Forrest GN, Mankes K, Jabra-Rizk MA, Weekes E, Johnson JK, Lincalis DP, Venezia RA (2006). Peptide nucleic acid fluorescence in situ hybridization-based identification of Candida albicans and its impact on mortality and antifungal therapy costs. J Clin Microbiol;44(9):3381-3.

Fout GS, Martinson BC, Moyer MW, Dahling DR (2003). A multiplex reverse transcription-PCR method for detection of human enteric viruses in groundwater. Appl Environ Microbiol;69(6):3158-64.

Fukuda S, Sasaki Y, Seno M (2008). Rapid and sensitive detection of norovirus genomes in oysters by a two-step isothermal amplification assay system combining nucleic acid sequence-based amplification and reverse transcription-loop-mediated isothermal amplification assays. Appl Environ Microbiol;74(12):3912-4.

Fumian TM, Leite JP, Marin VA, Miagostovich MP (2009). A rapid procedure for detecting noroviruses from cheese and fresh lettuce. J Virol Methods;155(1):39-43.

Gao Z, Tansil N (2009). A DNA biosensor based on the electrocatalytic oxidation of amine by a threading intercalator. Anal Chim Acta;636(1):77-82.

Gao Z, Agarwal A, Trigg AD, Singh N, Fang C, Tung CH, Fan Y, Buddharaju KD, Kong J (2007). Silicon nanowire arrays for label-free detection of DNA. Anal Chem;79(9):3291-7.

Gassilloud B, Huguet L, Maul A, Gantzer C (2007). Development of a viral concentration method for bottled water stored in hydrophobic support. J Virol Methods;142(1-2):98-104.

Gentry J, Vinjé J, Lipp EK (2009). A rapid and efficient method for quantitation of genogroups I and II norovirus from oysters and application in other complex environmental samples. J Virol Methods;156(1-2):59-65.

Germini A, Rossi S, Zanetti A, Corradini R, Fogher C, Marchelli R (2005). Development of a peptide nucleic acid array platform for the detection of genetically modified organisms in food. J Agric Food Chem;53(10):3958-62.

Germini A, Zanetti A, Salati C, Rossi S, Forré C, Schmid S, Marchelli R, Fogher C (2004a).Development of a seven-target multiplex PCR for the simultaneous detection of transgenic soybean and maize in feeds and foods. J Agric Food Chem;52(11):3275-80.

Germini A, Mezzelani A, Lesignoli F, Corradini R, Marchelli R, Bordoni R, Consolandi C, De Bellis G (2004b). Detection of genetically modified soybean using peptide nucleic acids (PNAs) and microarray technology. J Agric Food Chem;52(14):4535-40.

Gherna M, Merz WG (2009). Identification of Candida albicans and Candida glabrata within 1.5 hours directly from positive blood culture bottles with a shortened peptide nucleic acid fluorescence in situ hybridization protocol. J Clin Microbiol;47(1):247-8.

Gilgen M, Germann D, Lüthy J, Hübner P (1997). Three-step isolation method for sensitive detection of enterovirus, rotavirus, hepatitis A virus, and small round structured viruses in water samples. Int J Food Microbiol;37(2-3):189-99.

Gilje B, Heikkilä R, Oltedal S, Tjensvoll K, Nordgård O (2008). High-fidelity DNA polymerase enhances the sensitivity of a peptide nucleic acid clamp PCR assay for K-ras mutations. J Mol Diagn;10(4):325-31.

Gião MS, Wilks SA, Azevedo NF, Vieira MJ, Keevil CW (2009). Comparison between standard culture and peptide nucleic acid 16S rRNA hybridization quantification to study the influence of physico-chemical parameters on Legionella pneumophila survival in drinking water biofilms. Biofouling;25(4):343-51.

Gião MS, Wilks S, Azevedo NF, Vieira MJ, Keevil CW (2009). Incorporation of natural uncultivable Legionella pneumophila into potable water biofilms provides a protective niche against chlorination stress. Biofouling;25(4):335-41.

Good L, Nielsen PE (1998). Antisense inhibition of gene expression in bacteria by PNA targeted to mRNA. Nat Biotechnol. Apr;16(4):355-8.

Good L, Nielsen PE (1998). Inhibition of translation and bacterial growth by peptide nucleic acid targeted to ribosomal RNA. Proc Natl Acad Sci U S A;95(5):2073-6.

Govindaraju T, Kumar VA, Ganesh KN (2004a). Synthesis and evaluation of (1S,2R/1R,2S)-aminocyclohexylglycyl PNAs as conformationally preorganized PNA analogues for DNA/RNA recognition. J Org Chem;69(6):1858-65.

Govindaraju T, Kumar VA, Ganesh KN (2004b). (1S,2R/1R,2S)-cis-cyclopentyl PNAs (cpPNAs) as constrained PNA analogues: synthesis and evaluation of aeg-cpPNA chimera and stereopreferences in hybridization with DNA/RNA. J Org Chem;69(17):5725-34.

Govindaraju T, Kumar VA, Ganesh KN (2004c). cis-Cyclopentyl PNA (cpPNA) as constrained chiral PNA analogues: stereochemical dependence of DNA/RNA hybridization. Chem Commun;(7):860-1.

Govindaraju T, Gonnade RG, Bhadbhade MM, Kumar VA, Ganesh KN (2003). (1S,2R/1R,2S)-ainocyclohexyl glycyl thymine PNA: synthesis, monomer crystal structures, and DNA/RNA hybridization studies. Org Lett;5(17):3013-6.

Gray JJ, Kohli E, Ruggeri FM, Vennema H, Sánchez-Fauquier A, Schreier E, Gallimore CI, Iturriza-Gomara M, Giraudon H, Pothier P, Di Bartolo I, Inglese N, de Bruin E, van der Veer B, Moreno S, Montero V, de Llano MC, Höhne M, Diedrich SM (2007). European multicenter evaluation of commercial enzyme immunoassays for detecting norovirus antigen in fecal samples. Clin Vaccine Immunol;14(10):1349-55.

Green J, Wright PA, Gallimore CI, Mitchell O, Morgan-Capner P, Brown DW (1998). The role of environmental contamination with small round structured viruses in a hospital outbreak investigated by reverse-transcriptase polymerase chain reaction assay. J Hosp Infect;39(1):39-45.

Green J, Gallimore CI, Norcott JP, Lewis D, Brown DW (1995). Broadly reactive reverse transcriptase polymerase chain reaction for the diagnosis of SRSV-associated gastroenteritis. J Med Virol;47(4):392-8.

Green SM, Lambden PR, Caul EO, Clarke IN (1997). Capsid sequence diversity in small round structured viruses from recent UK outbreaks of gastroenteritis. J Med Virol;52(1):14-9.

Greene SR, Moe CL, Jaykus LA, Cronin M, Grosso L, Aarle P (2003). Evaluation of the NucliSens Basic Kit assay for detection of Norwalk virus RNA in stool specimens. J Virol Methods;108(1):123-31.

Grossmann TN, Strohbach A, Seitz O (2008). Achieving turnover in DNA-templated reactions. Chembiochem. Sep 22;9(14):2185-92.

Guévremont E, Brassard J, Houde A, Simard C, Trottier YL (2006). Development of an extraction and concentration procedure and comparison of RT-PCR primer systems for the detection of hepatitis A virus and norovirus GII in green onions. J Virol Methods;134(1-2):130-5.

Haaima G, Hansen HF, Christensen L, Dahl O, Nielsen PE (1997). Increased DNA binding and sequence discrimination of PNA oligomers containing 2,6-diaminopurine. Nucleic Acids Res;25(22):4639-43.

Häfliger D, Gilgen M, Lüthy J, Hübner P (1997). Seminested RT-PCR systems for small round structured viruses and detection of enteric viruses in seafood. Int J Food Microbiol;37(1):27-36.

Hagiwara T, Hattori J, Kaneda T (2006). PNA-in situ hybridization method for detection of HIV-1 DNA in virus-infected cells and subsequent detection of cellular and viral proteins. Methods Mol Biol.;326:139-49.

Hamilton SE, Simmons CG, Kathiriya IS, Corey DR (1999). Cellular delivery of peptide nucleic acids and inhibition of human telomerase. Chem Biol;6(6):343-51.

Hamza IA, Jurzik L, Stang A, Sure K, Uberla K, Wilhelm M (2009). Detection of human viruses in rivers of a densly-populated area in Germany using a virus adsorption elution method optimized for PCR analyses. Water Res;43(10):2657-68.

Haramoto E, Katayama H, Utagawa E, Ohgaki S (2009). Recovery of human norovirus from water by virus concentration methods. J Virol Methods;160(1-2):206-9.

Haramoto E, Katayama H, Oguma K, Ohgaki S (2005). Application of cation-coated filter method to detection of noroviruses, enteroviruses, adenoviruses, and torque teno viruses in the Tamagawa River in Japan. Appl Environ Microbiol;71(5):2403-11.

Hardy ME (2005). Norovirus protein structure and function. FEMS Microbiol Lett;253(1):1-8.

Harris JP, Edmunds WJ, Pebody R, Brown DW, Lopman BA (2008). Deaths from norovirus among the elderly, England and Wales. Emerg Infect Dis;14(10):1546-52.

Heitz F, Morris MC, Divita G (2009). Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. Br J Pharmacol;157(2):195-206.

Herbert B, Pitts AE, Baker SI, Hamilton SE, Wright WE, Shay JW, Corey DR (1999). Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. Proc Natl Acad Sci U S A;96(25):14276-81.

Hernandez-Morga J, Leon-Felix J, Peraza-Garay F, Gil-Salas BG, Chaidez C (2009). Detection and characterization of hepatitis A virus and Norovirus in estuarine water samples using ultrafiltration--RT-PCR integrated methods. J Appl Microbiol;106(5):1579-90.

Hirano T, Kuroda K, Kataoka M, Hayakawa Y(2009). Peptide-nucleic acids (PNAs) with pyrimido[4,5-d]pyrimidine-2,4,5,7-(1H,3H,6H,8H)-tetraone (PPT) as a universal base: their synthesis and binding affinity for oligodeoxyribonucleotides. Org Biomol Chem;7(14):2905-11.

Holland PM, Abramson RD, Watson R, Gelfand DH (1991). Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci U S A;88(16):7276-80.

Höhne M, Schreier E (2004). Detection and characterization of norovirus outbreaks in Germany: application of a one-tube RT-PCR using a fluorogenic real-time detection system. J Med Virol;72(2):312-9.

Hongmanee P, Stender H, Rasmussen OF (2001). Evaluation of a fluorescence in situ hybridization assay for differentiation between tuberculous and nontuberculous Mycobacterium species in smears of Lowenstein-Jensen and Mycobacteria Growth Indicator Tube cultures using peptide nucleic acid probes. J Clin Microbiol;39(3):1032-5.

Houde A, Leblanc D, Poitras E, Ward P, Brassard J, Simard C, Trottier YL (2006). Comparative evaluation of RT-PCR, nucleic acid sequence-based amplification (NASBA) and real-time RT-PCR for detection of noroviruses in faecal material. J Virol Methods;135(2):163-72.

Hsu CC, Riley LK, Livingston RS (2007). Molecular characterization of three novel murine noroviruses. Virus Genes;34(2):147-55.

Hu J, Matsui M, Gagnon KT, Schwartz JC, Gabillet S, Arar K, Wu J, Bezprozvanny I, Corey DR (2009). Allele-specific silencing of mutant huntingtin and ataxin-3 genes by targeting expanded CAG repeats in mRNAs. Nat Biotechnol;27(5):478-84.

Hymas W, Atkinson A, Stevenson J, Hillyard D (2007). Use of modified oligonucleotides to compensate for sequence polymorphisms in the real-time detection of norovirus. J Virol Methods;142(1-2):10-4.

Hyrup B, Nielsen PE (1996). Peptide nucleic acids (PNA): synthesis, properties and potential applications. Bioorg Med Chem;4(1):5-23.

Igloi GL (1998). Variability in the stability of DNA-peptide nucleic acid (PNA) single-base mismatched duplexes: real-time hybridization during affinity electrophoresis in PNA-containing gels. Proc Natl Acad Sci U S A;95(15):8562-7.

Ikeda H, Yoshida K, Ozeki M and Saito I (2001). Synthesis and characterization of flavin-tethered peptide nucleic acid. Tetrahedron Letters; 13, 2529-2531.

Ikeda H, Nakamura Y, Saito I (2002). Synthesis and characterization of naphthalimide-containing peptide nucleic acid. Tetrahedron Letters; 32, 5525-5528.

Imanaka M, Iida K, Nishizawa H, Fukuoka H, Takeno R, Takahashi K, Kaji H, Takahashi Y, Okimura Y, Kaji H, Imanishi Y, Chihara K (2007). McCune-Albright syndrome with acromegaly and fibrous dysplasia associated with the GNAS gene mutation identified by sensitive PNA-clamping method. Intern Med.;46(18):1577-83.

Isacsson J, Cao H, Ohlsson L, Nordgren S, Svanvik N, Westman G, Kubista M, Sjöback R, Sehlstedt U (2000). Rapid and specific detection of PCR products using light-up probes. Mol Cell Probes;14(5):321-8.

Ishida S, Yoshizumi S, Ikeda T, Miyoshi M, Okano M, Okui T (2008). Sensitive and rapid detection of norovirus using duplex TaqMan reverse transcription-polymerase chain reaction. J Med Virol;80(5):913-20.

Ito K, Katada H, Shigi N, Komiyama M (2009). Site-selective scission of human genome by artificial restriction DNA cutter. Chem Commun;(43):6542-4.

Ito S, Takeshita S, Nezu A, Aihara Y, Usuku S, Noguchi Y, Yokota S (2006). Norovirus-associated encephalopathy. Pediatr Infect Dis J;25(7):651-2.

Iturriza-Gómara M, Xerry J, Gallimore CI, Dockery C, Gray J (2008). Evaluation of the Loopamp (loop-mediated isothermal amplification) kit for detecting Norovirus RNA in faecal samples. J Clin Virol;42(4):389-93.

Iwamoto T, Sonobe T (2004). Peptide nucleic acid-mediated competitive PCR clamping for detection of rifampin-resistant Mycobacterium tuberculosis. Antimicrob Agents Chemother;48(10):4023-6.

Jarikote DV, Krebs N, Tannert S, Röder B, Seitz O (2007). Exploring base-pair-specific optical properties of the DNA stain thiazole orange. Chemistry.;13(1):300-10.

Jääskeläinen AJ, Maunula L (2006). Applicability of microarray technique for the detection of noro- and astroviruses. J Virol Methods;136(1-2):210-6.

Jean J, D'Souza DH, Jaykus LA (2004). Multiplex nucleic acid sequence-based amplification for simultaneous detection of several enteric viruses in model ready-to-eat foods. Appl Environ Microbiol;70(11):6603-10.

Jensen KK, Orum H, Nielsen PE, Nordén B (1997). Kinetics for hybridization of peptide nucleic acids (PNA) with DNA and RNA studied with the BIAcore technique. Biochemistry;36(16):5072-7.

Jeon B, Zhang Q (2009). Sensitization of Campylobacter jejuni to fluoroquinolone and macrolide antibiotics by antisense inhibition of the CmeABC multidrug efflux transporter. J Antimicrob Chemother;63(5):946-8.

Jiang X, Wang M, Wang K, Estes MK (1993). Sequence and genomic organization of Norwalk virus. Virology;195(1):51-61.

Jiang X, Wang J, Graham DY, Estes MK (1992). Detection of Norwalk virus in stool by polymerase chain reaction. J Clin Microbiol;30(10):2529-34.

Jones TH, Brassard J, Johns MW, Gagné MJ (2009). The effect of pre-treatment and sonication of centrifugal ultrafiltration devices on virus recovery. J Virol Methods;161(2):199-204.

Jothikumar N, Lowther JA, Henshilwood K, Lees DN, Hill VR, Vinjé J (2005). Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. Appl Environ Microbiol;71(4):1870-5.

Kageyama T, Kojima S, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Takeda N, Katayama K (2003). Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J Clin Microbiol;41(4):1548-57.

Kai E, Ikebukuro K, Hoshina S, Watanabe H, Karube I (2000). Detection of PCR products of Escherichia coli O157:H7 in human stool samples using surface plasmon resonance (SPR). FEMS Immunol Med Microbiol;29(4):283-8.

Kambhampati D, Nielsen PE, Knoll W (2001). Investigating the kinetics of DNA-DNA and PNA-DNA interactions using surface plasmon resonance-enhanced fluorescence spectroscopy. Biosens Bioelectron;16(9-12):1109-18.

Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Chanock RM (1972). Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. J Virol;10(5):1075-81.

Karim MR, Rhodes ER, Brinkman N, Wymer L, Fout GS (2009). New electropositive filter for concentrating enteroviruses and noroviruses from large volumes of water. Appl Environ Microbiol;75(8):2393-9.

Katada H, Komiyama M (2009). Artificial restriction DNA cutters as new tools for gene manipulation. Chembiochem;10(8):1279-88.

Katayama H, Haramoto E, Oguma K, Yamashita H, Tajima A, Nakajima H, Ohgaki S (2008). One-year monthly quantitative survey of noroviruses, enteroviruses, and adenoviruses in wastewater collected from six plants in Japan. Water Res;42(6-7):1441-8.

Katayama H, Shimasaki A, Ohgaki S (2002). Development of a virus concentration method and its application to detection of enterovirus and norwalk virus from coastal seawater. Appl Environ Microbiol;68(3):1033-9.

Katayama K, Shirato-Horikoshi H, Kojima S, Kageyama T, Oka T, Hoshino F, Fukushi S, Shinohara M, Uchida K, Suzuki Y, Gojobori T, Takeda N (2002). Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. Virology;299(2):225-239.

Kaufman SS, Chatterjee NK, Fuschino ME, Morse DL, Morotti RA, Magid MS, Gondolesi GE, Florman SS, Fishbein TM (2005). Characteristics of human calicivirus enteritis in intestinal transplant recipients. J Pediatr Gastroenterol Nutr;40(3):328-33.

Kaushik N, Basu A, Pandey VN (2002). Inhibition of HIV-1 replication by anti-trans-activation responsive polyamide nucleotide analog. Antiviral Res;56(1):13-27.

Keer JT, Birch L (2003). Molecular methods for the assessment of bacterial viability. J Microbiol Methods;53(2):175-83.

Khamrin P, Nguyen TA, Phan TG, Satou K, Masuoka Y, Okitsu S, Maneekarn N, Nishio O, Ushijima H (2008). Evaluation of immunochromatography and commercial enzyme-linked immunosorbent assay for rapid detection of norovirus antigen in stool samples. J Virol Methods;147(2):360-3.

Kim HY, Kwak IS, Hwang IG, Ko G (2008). Optimization of methods for detecting norovirus on various fruit. J Virol Methods;153(2):104-10.

Kimmel A and Oliver B (2006). DNA Microarrays Part A: Array Platforms & Wet-Bench Protocols. Academic Press, 2006.

Kimura H, Miyake K, Yamauchi Y, Nishiyama K, Iwata S, Iwatsuki K, Gotoh K, Kojima S, Ito Y, Nishiyama Y (2009). Identification of Epstein-Barr virus (EBV)-infected lymphocyte subtypes by flow cytometric in situ hybridization in EBV-associated lymphoproliferative diseases. J Infect Dis;200(7):1078-87.

Kingsley DH, Holliman DR, Calci KR, Chen H, Flick GJ (2007). Inactivation of a norovirus by high-pressure processing. Appl Environ Microbiol;73(2):581-5.

Kirishima T, Okanoue T, Daimon Y, Itoh Y, Nakamura H, Morita A, Toyama T, Minami M (2002). Detection of YMDD mutant using a novel sensitive method in chronic liver disease type B patients before and during lamivudine treatment. J Hepatol;37(2):259-65.

Kitajima M, Haramoto E, Phanuwan C, Katayama H, Ohgaki S (2009). Detection of genogroup IV norovirus in wastewater and river water in Japan. Lett Appl Microbiol;49(5):655-8.

Komiyama M, Aiba Y, Yamamoto Y, Sumaoka J. Artificial restriction DNA cutter for site-selective scission of double-stranded DNA with tunable scission site and specificity. Nat Protoc. 2008;3(4):655-62.

Kong J, Ferhan AR, Chen X, Zhang L (2008), Balasubramanian N. Polysaccharide templated silver nanowire for ultrasensitive electrical detection of nucleic acids. Anal Chem;80(19):7213-7.

Kou X, Wu Q, Zhang J, Fan H (2006). Rapid detection of noroviruses in fecal samples and shellfish by nucleic acid sequence-based amplification. J Microbiol;44(4):403-8.

Kroneman A, Verhoef L, Harris J, Vennema H, Duizer E, van Duynhoven Y, Gray J, Iturriza M, Böttiger B, Falkenhorst G, Johnsen C, von Bonsdorff CH, Maunula L, Kuusi M, Pothier P, Gallay A, Schreier E, Höhne M, Koch J, Szücs G, Reuter G, Krisztalovics K, Lynch M, McKeown P, Foley B, Coughlan S, Ruggeri FM, Di Bartolo I, Vainio K, Isakbaeva E, Poljsak-Prijatelj M, Grom AH, Mijovski JZ, Bosch A, Buesa J, Fauquier AS, Hernandéz-Pezzi G, Hedlund KO, Koopmans M (2008). Analysis of integrated virological and epidemiological reports of norovirus outbreaks collected within the Foodborne Viruses in Europe network from 1 July 2001 to 30 June 2006. J Clin Microbiol;46(9):2959-65.

Kroger K, Jung A, Reder S, Gauglitz G (2002). Versatile biosensor surface based on peptide nucleic acid with label free and total internal reflection fluorescence detection for quantification of endocrine disruptors. Analytica Chimica Acta; 469(1): 37-48.

Kuhn H, Demidov VV, Coull JM, Fiandaca MJ, Gildea BD, Frank-Kamenetskii MD (2002a). Hybridization of DNA and PNA molecular beacons to single-stranded and double-stranded DNA targets. J Am Chem Soc;124(6):1097-103.

Kuhn H, Demidov VV, Frank-Kamenetskii MD (2002b). Rolling-circle amplification under topological constraints. Nucleic Acids Res;30(2):574-80.

Kuhn H, Demidov VV, Gildea BD, Fiandaca MJ, Coull JC, Frank-Kamenetskii MD (2001). PNA beacons for duplex DNA. Antisense Nucleic Acid Drug Dev;11(4):265-70.

Kumar VA, Ganesh KN (2005). Conformationally constrained PNA analogues: structural evolution toward DNA/RNA binding selectivity. Acc Chem Res;38(5):404-12.

Köhler O, Jarikote DV, Seitz O (2005). Forced intercalation probes (FIT Probes): thiazole orange as a fluorescent base in peptide nucleic acids for homogeneous single-nucleotide-polymorphism detection. Chembiochem;6(1):69-77.

Köhler O, Seitz O (2003). Thiazole orange as fluorescent universal base in peptide nucleic acids. Chem Commun;(23):2938-9.

La Rosa G, Fontana S, Di Grazia A, Iaconelli M, Pourshaban M, Muscillo M (2007). Molecular identification and genetic analysis of Norovirus genogroups I and II in water environments: comparative analysis of different reverse transcription-PCR assays. Appl Environ Microbiol;73(13):4152-61.

Lambden PR, Caul EO, Ashley CR, Clarke IN (1993). Sequence and genome organization of a human small round-structured (Norwalk-like) virus. Science;259(5094):516-9.

Lambertini E, Spencer SK, Bertz PD, Loge FJ, Kieke BA, Borchardt MA (2008). Concentration of enteroviruses, adenoviruses, and noroviruses from drinking water by use of glass wool filters. Appl Environ Microbiol;74(10):2990-6.

Lamothe GT, Putallaz T, Joosten H, Marugg JD (2003). Reverse transcription-PCR analysis of bottled and natural mineral waters for the presence of noroviruses. Appl Environ Microbiol;69(11):6541-9.

Lansdorp PM, Verwoerd NP, van de Rijke FM, Dragowska V, Little MT, Dirks RW, Raap AK, Tanke HJ (1996). Heterogeneity in telomere length of human chromosomes. Hum Mol Genet;5(5):685-91.

Larsen RD, Schønau A, Thisted M, Petersen KH, Lohse J, Christensen B, Philip J, Pluzek KJ (2003). Detection of gamma-globin mRNA in fetal nucleated red blood cells by PNA fluorescence in situ hybridization. Prenat Diagn;23(1):52-9.

Lau LT, Fung YW, Yu AC (2006). Detection of animal viruses using nucleic acid sequence-based amplification (NASBA). Dev Biol (Basel).;126:7-15;

Laverick MA, Wyn-Jones AP, Carter MJ (2004). Quantitative RT-PCR for the enumeration of noroviruses (Norwalk-like viruses) in water and sewage. Lett Appl Microbiol.;39(2):127-36.

Le Guyader FS, Parnaudeau S, Schaeffer J, Bosch A, Loisy F, Pommepuy M, Atmar RL (2009). Detection and quantification of noroviruses in shellfish. Appl Environ Microbiol;75(3):618-24.

Le Guyader FS, Mittelholzer C, Haugarreau L, Hedlund KO, Alsterlund R, Pommepuy M, Svensson L (2004). Detection of noroviruses in raspberries associated with a gastroenteritis outbreak. Int J Food Microbiol;97(2):179-86.

Le Guyader F, Estes MK, Hardy ME, Neill FH, Green J, Brown DW, Atmar RL (1996). Evaluation of a degenerate primer for the PCR detection of human caliciviruses. Arch Virol. ;141(11):2225-35

Lee C, Kim SJ (2008). The genetic diversity of human noroviruses detected in river water in Korea. Water Res;42(17):4477-84.

Lee H, Jeon JH, Lim JC, Choi H, Yoon Y, Kim SK (2007). Peptide nucleic acid synthesis by novel amide formation. Org Lett;9(17):3291-3.

Lees D (2000). Viruses and bivalve shellfish. Int J Food Microbiol. Jul 25;59(1-2):81-116.

Lefmann M, Schweickert B, Buchholz P, Göbel UB, Ulrichs T, Seiler P, Theegarten D, Moter A (2006). Evaluation of peptide nucleic acid-fluorescence in situ hybridization for identification of clinically relevant mycobacteria in clinical specimens and tissue sections. J Clin Microbiol;44(10):3760-7.

Lehtola MJ, Torvinen E, Kusnetsov J, Pitkänen T, Maunula L, von Bonsdorff CH, Martikainen PJ, Wilks SA, Keevil CW, Miettinen IT (2007). Survival of Mycobacterium avium, Legionella pneumophila, Escherichia coli,

and caliciviruses in drinking water-associated biofilms grown under high-shear turbulent flow. Appl Environ Microbiol;73(9):2854-9.

Lehtola MJ, Torvinen E, Miettinen IT, Keevil CW (2006). Fluorescence in situ hybridization using peptide nucleic acid probes for rapid detection of Mycobacterium avium subsp. avium and Mycobacterium avium subsp. paratuberculosis in potable-water biofilms. Appl Environ Microbiol;72(1):848-53.

Leijon M, Mousavi-Jazi M, Kubista M (2006). LightUp probes in clinical diagnostics. Mol Aspects Med;27(2-3):160-75.

Leijon M, Gräslund A, Nielsen PE, Buchardt O, Nordén B, Kristensen SM, Eriksson M (1994). Structural characterization of PNA-DNA duplexes by NMR. Evidence for DNA in a B-like conformation. Biochemistry;33(33):9820-5.

Li M, Bhiladvala RB, Morrow TJ, Sioss JA, Lew KK, Redwing JM, Keating CD, Mayer TS (2008). Bottom-up assembly of large-area nanowire resonator arrays. Nat Nanotechnol;3(2):88-92.

Li Y, Di Naro E, Vitucci A, Zimmermann B, Holzgreve W, Hahn S (2005). Detection of paternally inherited fetal point mutations for beta-thalassemia using size-fractionated cell-free DNA in maternal plasma. JAMA. 2005 Feb 16;293(7):843-9.

Lietman SA, Ding C, Levine MA (2005). A highly sensitive polymerase chain reaction method detects activating mutations of the GNAS gene in peripheral blood cells in McCune-Albright syndrome or isolated fibrous dysplasia. J Bone Joint Surg Am;87(11):2489-94.

Lindesmith L, Moe C, Marionneau S, Ruvoen N, Jiang X, Lindblad L, Stewart P, LePendu J, Baric R (2003). Human susceptibility and resistance to Norwalk virus infection. Nat Med;9(5):548-53.

Liu B, Bazan GC (2005). Methods for strand-specific DNA detection with cationic conjugated polymers suitable for incorporation into DNA chips and microarrays. Proc Natl Acad Sci U S A;102(3):589-93.

Liu J, Wu Q, Kou X (2007). Development of a virus concentration method and its application for the detection of noroviruses in drinking water in China. J Microbiol;45(1):48-52.

Liu J, Tiefenauer L, Tian S, Nielsen PE, Knoll W (2006). PNA-DNA hybridization study using labeled streptavidin by voltammetry and surface plasmon fluorescence spectroscopy. Anal Chem;78(2):470-6.

Lodder WJ, de Roda Husman AM (2005). Presence of noroviruses and other enteric viruses in sewage and surface waters in The Netherlands. Appl Environ Microbiol;71(3):1453-61.

Lohse J, Dahl O, Nielsen PE (1999). Double duplex invasion by peptide nucleic acid: a general principle for sequence-specific targeting of double-stranded DNA. Proc Natl Acad Sci U S A;96(21):11804-8.

Lopman BA, Reacher MH, Vipond IB, Sarangi J, Brown DW (2004). Clinical manifestation of norovirus gastroenteritis in health care settings. Clin Infect Dis;39(3):318-24.

López-Andreo M, Garrido-Pertierra A, Puyet A (2006). Evaluation of post-polymerase chain reaction melting temperature analysis for meat species identification in mixed DNA samples. J Agric Food Chem;54(21):7973-8.

Luo JD, Chan EC, Shih CL, Chen TL, Liang Y, Hwang TL, Chiou CC (2006). Detection of rare mutant K-ras DNA in a single-tube reaction using peptide nucleic acid as both PCR clamp and sensor probe. Nucleic Acids Res;34(2):e12.

Luo X, Hsing IM (2009). Immobilization-free multiplex electrochemical DNA and SNP detection. Biosens Bioelectron;25(4):803-8.

Luo X, Lee TM, Hsing IM (2008). Immobilization-free sequence-specific electrochemical detection of DNA using ferrocene-labeled peptide nucleic acid. Anal Chem;80(19):7341-6.

MacKinnon KF, Qualley DF, Woski SA (2007). Polycyclic aromatic hydrocarbons as universal bases in peptide nucleic acid. Tetrahedron Letters; 45, 8074-8077.

Magulski T, Paulmann D, Bischoff B, Becker B, Steinmann E, Steinmann J, Goroncy-Bermes P, Steinmann J (2009). Inactivation of murine norovirus by chemical biocides on stainless steel. BMC Infect Dis;9:107.

Maldini M, Marzano FN, González-Fortes G, Papa R, Gandolfi G (2006). Fish and seafood traceability based on AFLP markers: elaboration of a species database . Aquaculture, 261, 487–494.

Marionneau S, Ruvoën N, Le Moullac-Vaidye B, Clement M, Cailleau-Thomas A, Ruiz-Palacois G, Huang P, Jiang X, Le Pendu J (2002). Norwalk virus binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals. Gastroenterology;122(7):1967-77.

Matsuda M, Suzuki F, Suzuki Y, Tsubota A, Akuta N, Hosaka T, Someya T, Kobayashi M, Saitoh S, Arase Y, Satoh J, Kobayashi M, Ikeda K, Miyakawa Y, Kumada H (2004). YMDD mutants in patients with chronic hepatitis B before treatment are not selected by lamivudine. J Med Virol;74(2):361-6.

Mattison K, Grudeski E, Auk B, Charest H, Drews SJ, Fritzinger A, Gregoricus N, Hayward S, Houde A, Lee BE, Pang XL, Wong J, Booth TF, Vinjé J (2009). Multicenter comparison of two norovirus ORF2-based genotyping protocols. J Clin Microbiol;47(12):3927-32.

Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV (1999). Food-related illness and death in the United States. Emerg Infect Dis;5(5):607-25.

Menchise V, De Simone G, Tedeschi T, Corradini R, Sforza S, Marchelli R, Capasso D, Saviano M, Pedone C (2003). Insights into peptide nucleic acid (PNA) structural features: the crystal structure of a D-lysine-based chiral PNA-DNA duplex. Proc Natl Acad Sci U S A;100(21):12021-6.

Medici MC, Martinelli M, Ruggeri FM, Abelli LA, Bosco S, Arcangeletti MC, Pinardi F, De Conto F, Calderaro A, Chezzi C, Dettori G (2005). Broadly reactive nested reverse transcription-PCR using an internal RNA standard control for detection of noroviruses in stool samples. J Clin Microbiol;43(8):3772-8.

Merrifield RB (1963). Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide. J Am Chem Soc; 85 (14), pp 2149–2154

Miagostovich MP, Ferreira FF, Guimarães FR, Fumian TM, Diniz-Mendes L, Luz SL, Silva LA, Leite JP (2008). Molecular detection and characterization of gastroenteritis viruses occurring naturally in the stream waters of Manaus, central Amazonia, Brazil. Appl Environ Microbiol;74(2):375-82.

Miyazawa H, Tanaka T, Nagai Y, Matsuoka M; Huqun, Sutani A, Udagawa K, Zhang J, Hirama T, Murayama Y, Koyama N, Ikebuchi K, Nagata M, Kanazawa M, Nukiwa T, Takenoshita S, Kobayashi K, Hagiwara K (2008). Peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp-based detection test for gefitinib-refractory T790M epidermal growth factor receptor mutation. Cancer Sci;99(3):595-600.

Moe CL, Gentsch J, Ando T, Grohmann G, Monroe SS, Jiang X, Wang J, Estes MK, Seto Y, Humphrey C (1994). Application of PCR to detect Norwalk virus in fecal specimens from outbreaks of gastroenteritis. J Clin Microbiol;32(3):642-8.

Mohamed N, Belák S, Hedlund KO, Blomberg J (2006). Experience from the development of a diagnostic single tube real-time PCR for human caliciviruses, Norovirus genogroups I and II. J Virol Methods;132(1-2):69-76.

Moore C, Clark EM, Gallimore CI, Corden SA, Gray JJ, Westmoreland D (2004). Evaluation of a broadly reactive nucleic acid sequence based amplification assay for the detection of noroviruses in faecal material. J Clin Virol;29(4):290-6.

Mori Y, Notomi T (2009). Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. J Infect Chemother;15(2):62-9.

Morton V, Jean J, Farber J, Mattison K (2009). Detection of noroviruses in ready-to-eat foods by using carbohydrate-coated magnetic beads. Appl Environ Microbiol;75(13):4641-3.

Mun HY, Girigoswami A, Jung C, Cho DY, Park HG (2009). SNPs detection by a single-strand specific nuclease on a PNA zip-code microarray. Biosens Bioelectron;24(6):1706-11.

Munakata K, Iwamoto K, Bundo M, Kato T (2005). Mitochondrial DNA 3243A>G mutation and increased expression of LARS2 gene in the brains of patients with bipolar disorder and schizophrenia. Biol Psychiatry;57(5):525-32.

Murdock DG, Christacos NC, Wallace DC (2000). The age-related accumulation of a mitochondrial DNA control region mutation in muscle, but not brain, detected by a sensitive PNA-directed PCR clamping based method. Nucleic Acids Res;28(21):4350-5.

Mutoh K, Hakamata A, Yagi H, Kurokawa K, Miki N, Kurita I (2009). Evaluation of new commercial immunochromatography kit for norovirus in feces. Pediatr Int;51(1):164-6.

Nagai Y, Miyazawa H, Huqun, Tanaka T, Udagawa K, Kato M, Fukuyama S, Yokote A, Kobayashi K, Kanazawa M, Hagiwara K (2005). Genetic heterogeneity of the epidermal growth factor receptor in non-small cell lung cancer cell lines revealed by a rapid and sensitive detection system, the peptide nucleic acid-locked nucleic acid PCR clamp. Cancer Res;65(16):7276-82.

Nielsen PE, Egholm M, Berg RH, Buchardt O (1991). Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science;254(5037):1497-500.

P.E. Nielsen (2004) Peptide Nucleic Acids: Protocols and Applications (2nd ed.), Horizon Bioscience, Norfolk, UK (2004)

Nordgren J, Matussek A, Mattsson A, Svensson L, Lindgren PE (2009). Prevalence of norovirus and factors influencing virus concentrations during one year in a full-scale wastewater treatment plant. Water Res;43(4):1117-25.

Nordgren J, Bucardo F, Dienus O, Svensson L, Lindgren PE (2008). Novel light-upon-extension real-time PCR assays for detection and quantification of genogroup I and II noroviruses in clinical specimens. J Clin Microbiol;46(1):164-70.

Ogata N, Ichida T, Aoyagi Y, Kitajima I (2003). Development of peptide nucleic acid mediated polymerase chain reaction clamping (PMPC)--direct sequencing method for detecting lamivudine-resistant hepatitis B virus (HBV) variants with high sensitivity and specificity. Rinsho Byori;51(4):313-9.

Ohishi W, Shirakawa H, Kawakami Y, Kimura S, Kamiyasu M, Tazuma S, Nakanishi T, Chayama K (2004). Identification of rare polymerase variants of hepatitis B virus using a two-stage PCR with peptide nucleic acid clamping. J Med Virol;72(4):558-65.

Ohishi W, Chayama K (2003). Rare quasispecies in the YMDD motif of hepatitis B virus detected by polymerase chain reaction with peptide nucleic acid clamping. Intervirology;46(6):355-61.

Okamoto A, Tanabe K, Saito I (2001). Synthesis and properties of peptide nucleic acids containing a psoralen unit. Org Lett;3(6):925-7.

Oliveira K, Haase G, Kurtzman C, Hyldig-Nielsen JJ, Stender H (2001). Differentiation of Candida albicans and Candida dubliniensis by fluorescent in situ hybridization with peptide nucleic acid probes. J Clin Microbiol;39(11):4138-41.

Ortiz E, Estrada G, Lizardi PM (1998). PNA molecular beacons for rapid detection of PCR amplicons. Mol Cell Probes;12(4):219-26.

Orum H (2000). PCR clamping. Curr Issues Mol Biol;2(1):27-30.

Orum H, Nielsen PE, Jørgensen M, Larsson C, Stanley C, Koch T (1995). Sequence-specific purification of nucleic acids by PNA-controlled hybrid selection. Biotechniques;19(3):472-80.

Oyama M, Ikeda T, Lim T, Ikebukuro K, Masuda Y, Karube I (2000-2001). Detection of toxic chemicals with high sensitivity by measuring the quantity of induced P450 mRNAs based on surface plasmon resonance. Biotechnol Bioeng.;71(3):217-22.

Padilla E, Manterola JM, Rasmussen OF, Lonca J, Domínguez J, Matas L, Hernández A, Ausina V (2000). Evaluation of a fluorescence hybridisation assay using peptide nucleic acid probes for identification and differentiation of tuberculous and non-tuberculous mycobacteria in liquid cultures. Eur J Clin Microbiol Infect Dis;19(2):140-5.

Pagotto F, Corneau N, Mattison K, Bidawid S (2008). Development of a DNA microarray for the simultaneous detection and genotyping of noroviruses. J Food Prot;71(7):1434-41.

Panagene website: http://www.panagene.com/main.php

Pandey VN, Upadhyay A, Chaubey B (2009). Prospects for antisense peptide nucleic acid (PNA) therapies for HIV. Expert Opin Biol Ther;9(8):975-89.

Pang X, Lee B, Chui L, Preiksaitis JK, Monroe SS (2004). Evaluation and validation of real-time reverse transcription-pcr assay using the LightCycler system for detection and quantitation of norovirus. J Clin Microbiol;42(10):4679-85.

Pang XL, Preiksaitis JK, Lee B (2005). Multiplex real time RT-PCR for the detection and quantitation of norovirus genogroups I and II in patients with acute gastroenteritis. J Clin Virol;33(2):168-71.

Parashar UD, Monroe SS (2001). "Norwalk-like viruses" as a cause of foodborne disease outbreaks. Rev Med Virol;11(4):243-52.

Parida M, Sannarangaiah S, Dash PK, Rao PV, Morita K (2008). Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. Rev Med Virol;18(6):407-21.

Park H., Germini A., Sforza S., Corradini R., Marchelli R., Knoll W (2006). Kinetic and affinity analyses of hybridization reactions between peptide nucleic acid probes and DNA targets using surface plasmon field-enhanced fluorescence spectroscopy. Biointerphases 1, 4, 113-122.

Park H., Germini A., Sforza S., Corradini R., Marchelli R., Knoll W (2007). Effect of ionic strength on PNA-DNA hybridization on surfaces and in solution. Biointerphases, 2, 2, 80-88.

Patel MM, Hall AJ, Vinjé J, Parashar UD (2009). Noroviruses: a comprehensive review. J Clin Virol;44(1):1-8.

Patel MM, Widdowson MA, Glass RI, Akazawa K, Vinjé J, Parashar UD (2008). Systematic literature review of role of noroviruses in sporadic gastroenteritis. Emerg Infect Dis;14(8):1224-31.

Patterson SS, Smith MW, Casper ET, Huffman D, Stark L, Fries D, Paul JH (2006). A nucleic acid sequence-based amplification assay for real-time detection of norovirus genogroup II. J Appl Microbiol;101(4):956-63.

Peano C, Lesignoli F, Gulli M, Corradini R, Samson MC, Marchelli R, Marmiroli N (2005). Development of a peptide nucleic acid polymerase chain reaction clamping assay for semiquantitative evaluation of genetically modified organism content in food. Anal Biochem;344(2):174-82.

Peeters JK, Van der Spek PJ (2005). Growing applications and advancements in microarray technology and analysis tools. Cell Biochem Biophys.;43(1):149-66.

Pellestor F (2006). In situ aneuploidy assessment in human sperm: the use of primed in situ and peptide nucleic acid-fluorescence in situ hybridization techniques. Asian J Androl. Jul;8(4):387-92.

Perry-O'Keefe H, Rigby S, Oliveira K, Sørensen D, Stender H, Coull J, Hyldig-Nielsen JJ (2001). Identification of indicator microorganisms using a standardized PNA FISH method. J Microbiol Methods;47(3):281-92.

Pesce CD, Bolacchi F, Bongiovanni B, Cisotta F, Capozzi M, Diviacco S, Quadrifoglio F, Mango R, Novelli G, Mossa G, Esposito C, Ombres D, Rocchi G, Bergamini A (2005). Anti-gene peptide nucleic acid targeted to proviral HIV-1 DNA inhibits in vitro HIV-1 replication. Antiviral Res;66(1):13-22.

Pession A, Tonelli R, Fronza R, Sciamanna E, Corradini R, Sforza S, Tedeschi T, Marchelli R, Montanaro L, Camerin C, Franzoni M, Paolucci G (2004). Targeted inhibition of NMYC by peptide nucleic acid in N-myc amplified human neuroblastoma cells: cell-cycle inhibition with induction of neuronal cell differentiation and apoptosis. Int J Oncol;24(2):265-72.

Petersen K, Vogel U, Rockenbauer E, Nielsen KV, Kølvraa S, Bolund L, Nexø B (2004). Short PNA molecular beacons for real-time PCR allelic discrimination of single nucleotide polymorphisms. Mol Cell Probes;18(2):117-22.

Petrinca AR, Donia D, Pierangeli A, Gabrieli R, Degener AM, Bonanni E, Diaco L, Cecchini G, Anastasi P, Divizia M (2009). Presence and environmental circulation of enteric viruses in three different wastewater treatment plants. J Appl Microbiol;106(5):1608-17.

Pianowski Z, Gorska K, Oswald L, Merten CA, Winssinger N (2009). Imaging of mRNA in live cells using nucleic acid-templated reduction of azidorhodamine probes. J Am Chem Soc;131(18):6492-7.

Pianowski ZL, Winssinger N (2007). Fluorescence-based detection of single nucleotide permutation in DNA via catalytically templated reaction. Chem Commun (Camb);(37):3820-2.

Pokorski JK, Nam JM, Vega RA, Mirkin CA, Appella DH (2005). Cyclopentane-modified PNA improves the sensitivity of nanoparticle-based scanometric DNA detection. Chem Commun;(16):2101-3.

Popping B (2002). The application of biotechnological methods in authenticity testing. J Biotechnol; 98(1):107-12.

Potter AJ, Wener MH (2005). Flow cytometric analysis of fluorescence in situ hybridization with dye dilution and DNA staining (flow-FISH-DDD) to determine telomere length dynamics in proliferating cells. Cytometry A68(1):53-8.

Pouchain D, Díaz-Mochón JJ, Bialy L, Bradley M (2007). A 10,000 member PNA-encoded peptide library for profiling tyrosine kinases. ACS Chem Biol;2(12):810-8.

Prasad BV, Hardy ME, Dokland T, Bella J, Rossmann MG, Estes MK (1999). X-ray crystallographic structure of the Norwalk virus capsid. Science;286(5438):287-90.

Prescott AM, Fricker CR (1999). Use of PNA oligonucleotides for the in situ detection of Escherichia coli in water. Mol Cell Probes;13(4):261-8.

Pusch D, Oh DY, Wolf S, Dumke R, Schröter-Bobsin U, Höhne M, Röske I, Schreier E (2005). Detection of enteric viruses and bacterial indicators in German environmental waters. Arch Virol;150(5):929-47.

Ramirez S, Giammanco GM, De Grazia S, Colomba C, Martella V, Arista S (2009). Emerging GII.4 norovirus variants affect children with diarrhea in Palermo, Italy in 2006. J Med Virol;81(1):139-45.

Raymond FR, Ho HA, Peytavi R, Bissonnette L, Boissinot M, Picard FJ, Leclerc M, Bergeron MG (2005). Detection of target DNA using fluorescent cationic polymer and peptide nucleic acid probes on solid support. BMC Biotechnol; 5:10.

Rasmussen H, Kastrup JS, Nielsen JN, Nielsen JM, Nielsen PE (1997). Crystal structure of a peptide nucleic acid (PNA) duplex at 1.7 A resolution. Nat Struct Biol;4(2):98-101.

Reisberg S, Dang LA, Nguyen QA, Piro B, Noel V, Nielsen PE, Le LA, Pham MC (2008). Label-free DNA electrochemical sensor based on a PNA-functionalized conductive polymer. Talanta;76(1):206-10.

Reller ME, Mallonee AB, Kwiatkowski NP, Merz WG (2007). Use of peptide nucleic acid-fluorescence in situ hybridization for definitive, rapid identification of five common Candida species. J Clin Microbiol;45(11):3802-3.

Richards GP, Watson MA, Kingsley DH (2004a). A SYBR green, real-time RT-PCR method to detect and quantitate Norwalk virus in stools. J Virol Methods;116(1):63-70

Richards GP, Watson MA, Fankhauser RL, Monroe SS (2004b). Genogroup I and II noroviruses detected in stool samples by real-time reverse transcription-PCR using highly degenerate universal primers. Appl Environ Microbiol;70(12):7179-84. .

Rigby S, Procop GW, Haase G, Wilson D, Hall G, Kurtzman C, Oliveira K, Von Oy S, Hyldig-Nielsen JJ, Coull J, Stender H (2002). Fluorescence in situ hybridization with peptide nucleic acid probes for rapid identification of Candida albicans directly from blood culture bottles. J Clin Microbiol;40(6):2182-6.

Rockx B, De Wit M, Vennema H, Vinjé J, De Bruin E, Van Duynhoven Y, Koopmans M (2002). Natural history of human calicivirus infection: a prospective cohort study. Clin Infect Dis;35(3):246-53.

Rolfe KJ, Parmar S, Mururi D, Wreghitt TG, Jalal H, Zhang H, Curran MD (2007). An internally controlled, one-step, real-time RT-PCR assay for norovirus detection and genogrouping. J Clin Virol;39(4):318-21.

Rossi S, Scaravelli E, Germini A, Corradini R, Fogher C, Marchelli R (2006). A PNA-array platform for the detection of hidden allergens in foodstuffs. European Food Research and Technology. Eur. Food Res. Technol., *223*, 1–6.

Rossi S, 2007: PhD thesis

Russell VJ, Hold GL, Pryde SE, Rehbein H, Quinteiro J, Rey-Mendez M, Sotelo CG, Pérez-Martin RI, Santos AT, Rosa C (2000). Use of restriction fragment length polymorphism to distinguish between salmon species. J Agric Food Chem;48(6):2184-8.

Rutjes SA, van den Berg HH, Lodder WJ, de Roda Husman AM (2006a). Real-time detection of noroviruses in surface water by use of a broadly reactive nucleic acid sequence-based amplification assay. Appl Environ Microbiol;72(8):5349-58.
Rutjes SA, Lodder-Verschoor F, van der Poel WH, van Duijnhoven YT, de Roda Husman AM (2006b). Detection of noroviruses in foods: a study on virus extraction procedures in foods implicated in outbreaks of human gastroenteritis. J Food Prot;69(8):1949-56.

Rzezutka A, Alotaibi M, D'Agostino M, Cook N (2005). A centrifugation-based method for extraction of norovirus from raspberries. J Food Prot;68(9):1923-5.

Sato Y, Ikegaki S, Suzuki K, Kawaguchi H (2003). Hydrogel-microsphere-enhanced surface plasmon resonance for the detection of a K-ras point mutation employing peptide nucleic acid. J Biomater Sci Polym Ed.;14(8):803-20.

Sazani P, Gemignani F, Kang SH, Maier MA, Manoharan M, Persmark M, Bortner D, Kole R (2002). Systemically delivered antisense oligomers upregulate gene expression in mouse tissues. Nat Biotechnol;20(12):1228-33.

Schmid M, Oehme R, Schalasta G, Brockmann S, Kimmig P, Enders G (2004). Fast detection of Noroviruses using a real-time PCR assay and automated sample preparation. BMC Infect Dis;4:15.

Schwab KJ, Neill FH, Fankhauser RL, Daniels NA, Monroe SS, Bergmire-Sweat DA, Estes MK, Atmar RL (2000). Development of methods to detect "Norwalk-like viruses" (NLVs) and hepatitis A virus in delicatessen foods: application to a food-borne NLV outbreak. Appl Environ Microbiol;66(1):213-8.

Schwab KJ, Estes MK, Neill FH, Atmar RL (1997). Use of heat release and an internal RNA standard control in reverse transcription-PCR detection of Norwalk virus from stool samples. J Clin Microbiol;35(2):511-4.

Seeger C, Batz HG, Orum H (1997). PNA-mediated purification of PCR amplifiable human genomic DNA from whole blood. Biotechniques;23(3):512-7.

Seitz O (2000). Solid-Phase Synthesis of Doubly Labeled Peptide Nucleic Acids as Probes for the Real-Time Detection of Hybridization This work was supported by the Fonds der Chemischen Industrie. Angew Chem Int Ed Engl;39(18):3249-3252.

Seitz O, Bergmann F, Heindl D (1999). A Convergent Strategy for the Modification of Peptide Nucleic Acids: Novel Mismatch-Specific PNA-Hybridization Probes. Angew Chem Int Ed Engl;38(15):2203-2206.

Selvaraju SB, Kapoor R, Yadav JS (2008). Peptide nucleic acid-fluorescence in situ hybridization (PNA-FISH) assay for specific detection of Mycobacterium immunogenum and DNA-FISH assay for analysis of pseudomonads in metalworking fluids and sputum. Mol Cell Probes;22(5-6):273-80.

Sen A, Nielsen PE (2007). On the stability of peptide nucleic acid duplexes in the presence of organic solvents. Nucleic Acids Res;35(10):3367-74.

Sforza S, Scaravelli E, Corradini R, Marchelli R (2005). Unconventional method based on circular dichroism to detect peanut DNA in food by means of a PNA probe and a cyanine dye. Chirality;17(9):515-21.

Shepard JR, Addison RM, Alexander BD, Della-Latta P, Gherna M, Haase G, Hall G, Johnson JK, Merz WG, Peltroche-Llacsahuanga H, Stender H, Venezia RA, Wilson D, Procop GW, Wu F, Fiandaca MJ (2008). Multicenter evaluation of the Candida albicans/Candida glabrata peptide nucleic acid fluorescent in situ hybridization method for simultaneous dual-color identification of C. albicans and C. glabrata directly from blood culture bottles. J Clin Microbiol;46(1):50-5.

Siebenga JJ, Beersma MF, Vennema H, van Biezen P, Hartwig NJ, Koopmans M (2008). High prevalence of prolonged norovirus shedding and illness among hospitalized patients: a model for in vivo molecular evolution. J Infect Dis. 2008 Oct 1;198(7):994-1001. Erratum in: J Infect Dis;198(10):1575.

Skraber S, Ogorzaly L, Helmi K, Maul A, Hoffmann L, Cauchie HM, Gantzer C (2009). Occurrence and persistence of enteroviruses, noroviruses and F-specific RNA phages in natural wastewater biofilms. Water Res;43(19):4780-9.

Skraber S, Gantzer C, Helmi K, Hoffmann L, Cauchie HM (2009). Simultaneous Concentration of Enteric Viruses and Protozoan Parasites: A Protocol Based on Tangential Flow Filtration and Adapted to Large Volumes of Surface and Drinking Waters. Food and Environmental Virology; 1(2): 66-76.

Smolina I, Lee C, Frank-Kamenetskii M (2007). Detection of low-copy-number genomic DNA sequences in individual bacterial cells by using peptide nucleic acid-assisted rolling-circle amplification and fluorescence in situ hybridization. Appl Environ Microbiol;73(7):2324-8.

Smolina IV, Kuhn H, Lee C, Frank-Kamenetskii MD (2008). Fluorescence-based detection of short DNA sequences under non-denaturing conditions. Bioorg Med Chem;16(1):84-93.

Smolina IV, Demidov VV, Cantor CR, Broude NE (2004). Real-time monitoring of branched rolling-circle DNA amplification with peptide nucleic acid beacon. Anal Biochem;335(2):326-9.

Socher E, Jarikote DV, Knoll A, Röglin L, Burmeister J, Seitz O (2008a). FIT probes: peptide nucleic acid probes with a fluorescent base surrogate enable real-time DNA quantification and single nucleotide polymorphism discovery. Anal Biochem;375(2):318-30.

Socher E, Bethge L, Knoll A, Jungnick N, Herrmann A, Seitz O (2008b). Low-noise stemless PNA beacons for sensitive DNA and RNA detection. Angew Chem Int Ed Engl.;47(49):9555-9.

Søgaard M, Stender H, Schønheyder HC (2005). Direct identification of major blood culture pathogens, including Pseudomonas aeruginosa and Escherichia coli, by a panel of fluorescence in situ hybridization assays using peptide nucleic acid probes. J Clin Microbiol;43(4):1947-9.

Song JY, Park HG, Jung SO, Park J (2005). Diagnosis of HNF-1alpha mutations on a PNA zip-code microarray by single base extension. Nucleic Acids Res;33(2):e19.

Stals A, Baert L, Botteldoorn N, Werbrouck H, Herman L, Uyttendaele M, Van Coillie E (2009). Multiplex real-time RT-PCR for simultaneous detection of GI/GII noroviruses and murine norovirus 1. J Virol Methods;161(2):247-53.

Stender H, Kurtzman C, Hyldig-Nielsen JJ, Sørensen D, Broomer A, Oliveira K, Perry-O'Keefe H, Sage A, Young B, Coull J (2001). Identification of Dekkera bruxellensis (Brettanomyces) from wine by fluorescence in situ hybridization using peptide nucleic acid probes. Appl Environ Microbiol;67(2):938-41.

Stender H, Broomer AJ, Oliveira K, Perry-O'Keefe H, Hyldig-Nielsen JJ, Sage A, Coull J (2001). Rapid detection, identification, and enumeration of Escherichia coli cells in municipal water by chemiluminescent in situ hybridization. Appl Environ Microbiol;67(1):142-7.

Stender H, Broomer A, Oliveira K, Perry-O'Keefe H, Hyldig-Nielsen JJ, Sage A, Young B, Coull J (2000). Rapid detection, identification, and enumeration of Pseudomonas aeruginosa in bottled water using peptide nucleic acid probes. J Microbiol Methods;42(3):245-53.

Stender H, Lund K, Petersen KH, Rasmussen OF, Hongmanee P, Miörner H, Godtfredsen SE (1999). Fluorescence In situ hybridization assay using peptide nucleic acid probes for differentiation between tuberculous and nontuberculous mycobacterium species in smears of mycobacterium cultures. J Clin Microbiol;37(9):2760-5.

Straub TM, Höner zu Bentrup K, Orosz-Coghlan P, Dohnalkova A, Mayer BK, Bartholomew RA, Valdez CO, Bruckner-Lea CJ, Gerba CP, Abbaszadegan M, Nickerson CA (2007). In vitro cell culture infectivity assay for human noroviruses. Emerg Infect Dis;13(3):396-403.

Su X, Teh HF, Aung KM, Zong Y, Gao Z (2008). Femtomol SPR detection of DNA-PNA hybridization with the assistance of DNA-guided polyaniline deposition. Biosens Bioelectron;23(11):1715-20.

Sutani A, Nagai Y, Udagawa K, Uchida Y, Koyama N, Murayama Y, Tanaka T, Miyazawa H, Nagata M, Kanazawa M, Hagiwara K, Kobayashi K (2006). Gefitinib for non-small-cell lung cancer patients with epidermal growth factor receptor gene mutations screened by peptide nucleic acid-locked nucleic acid PCR clamp. Br J Cancer;95(11):1483-9.

Svanvik N, Westman G, Wang D, Kubista M (2000). Light-up probes: thiazole orange-conjugated peptide nucleic acid for detection of target nucleic acid in homogeneous solution. Anal Biochem;281(1):26-35.

Svanvik N, Ståhlberg A, Sehlstedt U, Sjöback R, Kubista M (2000b). Detection of PCR products in real time using light-up probes. Anal Biochem;287(1):179-82.

Taback B, Bilchik AJ, Saha S, Nakayama T, Wiese DA, Turner RR, Kuo CT, Hoon DS (2004). Peptide nucleic acid clamp PCR: a novel K-ras mutation detection assay for colorectal cancer micrometastases in lymph nodes. Int J Cancer;111(3):409-14.

Tajiri-Utagawa E, Hara M, Takahashi K, Watanabe M, Wakita T (2009). Development of a rapid high-throughput method for high-resolution melting analysis for routine detection and genotyping of noroviruses. J Clin Microbiol;47(2):435-40.

Takanashi S, Okame M, Shiota T, Takagi M, Yagyu F, Tung PG, Nishimura S, Katsumata N, Igarashi T, Okitsu S, Ushijima H (2008). Development of a rapid immunochromatographic test for noroviruses genogroups I and II. J Virol Methods;148(1-2):1-8.

Tanaka T, Nagai Y, Miyazawa H, Koyama N, Matsuoka S, Sutani A, Huqun, Udagawa K, Murayama Y, Nagata M, Shimizu Y, Ikebuchi K, Kanazawa M, Kobayashi K, Hagiwara K (2007). Reliability of the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp-based test for epidermal growth factor receptor mutations integrated into the clinical practice for non-small cell lung cancers. Cancer Sci;98(2):246-52.

Taneja KL, Chavez EA, Coull J, Lansdorp PM (2001). Multicolor fluorescence in situ hybridization with peptide nucleic acid probes for enumeration of specific chromosomes in human cells. Genes Chromosomes Cancer;30(1):57-63.

Tedeschi T, Chiari M, Galaverna G, Sforza S, Cretich M, Corradini R, Marchelli R (2005). Detection of the R553X DNA single point mutation related to cystic fibrosis by a "chiral box" D-lysine-peptide nucleic acid probe by capillary electrophoresis. Electrophoresis;26(22):4310-6.

Teunis PF, Moe CL, Liu P, Miller SE, Lindesmith L, Baric RS, Le Pendu J, Calderon RL (2008). Norwalk virus: how infectious is it? J Med Virol;80(8):1468-76.

Thèves C, Keyser-Tracqui C, Crubézy E, Salles JP, Ludes B, Telmon N (2006). Detection and quantification of the age-related point mutation A189G in the human mitochondrial DNA. J Forensic Sci;51(4):865-73.

Todt S, Blohm DH (2009). Immobilization chemistries. Methods Mol Biol.;529:81-100.

Tomac S, Sarkar M, Ratilainen T, Wittung P, Nielsen PE, Nordén B, Gräslund A (1996). Ionic Effects on the Stability and Conformation of Peptide Nucleic Acid Complexes. Journal of the American Chemical Society; *118* (24), 5544-5552.

Tonelli R, Purgato S, Camerin C, Fronza R, Bologna F, Alboresi S, Franzoni M, Corradini R, Sforza S, Faccini A, Shohet JM, Marchelli R, Pession A (2005). Anti-gene peptide nucleic acid specifically inhibits MYCN expression in human neuroblastoma cells leading to cell growth inhibition and apoptosis. Mol Cancer Ther;4(5):779-86.

Totsingan F, Rossi S, Corradini R, Tedeschi T, Sforza S, Juris A, Scaravelli E, Marchelli R (2008). Label-free selective DNA detection with high mismatch recognition by PNA beacons and ion exchange HPLC. Org Biomol Chem;6(7):1232-7.

Trujillo AA, McCaustland KA, Zheng DP, Hadley LA, Vaughn G, Adams SM, Ando T, Glass RI, Monroe SS (2006). Use of TaqMan real-time reverse transcription-PCR for rapid detection, quantification, and typing of norovirus. J Clin Microbiol;44(4):1405-12.

Turcios-Ruiz RM, Axelrod P, St John K, Bullitt E, Donahue J, Robinson N, Friss HE (2008). Outbreak of necrotizing enterocolitis caused by norovirus in a neonatal intensive care unit. J Pediatr;153(3):339-44.

Tyagi S, Kramer FR (1996). Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol;14(3):303-8.

Urata M, Wada Y, Kim SH, Chumpia W, Kayamori Y, Hamasaki N, Kang D (2004). High-sensitivity detection of the A3243G mutation of mitochondrial DNA by a combination of allele-specific PCR and peptide nucleic acid-directed PCR clamping. Clin Chem;50(11):2045-51.

Victoria M, Guimarães F, Fumian T, Ferreira F, Vieira C, Leite JP, Miagostovich M (2009). Evaluation of an adsorption-elution method for detection of astrovirus and norovirus in environmental waters. J Virol Methods;156(1-2):73-6.

Vinjé J, Vennema H, Maunula L, von Bonsdorff CH, Hoehne M, Schreier E, Richards A, Green J, Brown D, Beard SS, Monroe SS, de Bruin E, Svensson L, Koopmans MP (2003). International collaborative study to compare reverse transcriptase PCR assays for detection and genotyping of noroviruses. J Clin Microbiol;41(4):1423-33.

Volpi EV, Bridger JM (2008). FISH glossary: an overview of the fluorescence in situ hybridization technique. Biotechniques;45(4):385-6, 388, 390 passim.

von Wintzingerode F, Landt O, Ehrlich A, Göbel UB (2000). Peptide nucleic acid-mediated PCR clamping as a useful supplement in the determination of microbial diversity. Appl Environ Microbiol;66(2):549-57.

Wang J, Jiang X, Madore HP, Gray J, Desselberger U, Ando T, Seto Y, Oishi I, Lew JF, Green KY, et al (1994). Sequence diversity of small, round-structured viruses in the Norwalk virus group. J Virol;68(9):5982-90.

Wang QH, Costantini V, Saif LJ (2007). Porcine enteric caliciviruses: genetic and antigenic relatedness to human caliciviruses, diagnosis and epidemiology. Vaccine;25(30):5453-66.

Wang Y, Chen M, Zhang L, Ding Y, Luo Y, Xu Q, Shi J, Cao L, Fu W (2009). Rapid detection of human papilloma virus using a novel leaky surface acoustic wave peptide nucleic acid biosensor. Biosens Bioelectron;24(12):3455-60.

Weile J, Knabbe C (2009). Current applications and future trends of molecular diagnostics in clinical bacteriology. Anal Bioanal Chem;394(3):731-42.

Westhoff TH, Vergoulidou M, Loddenkemper C, Schwartz S, Hofmann J, Schneider T, Zidek W, van der Giet M (2009). Chronic norovirus infection in renal transplant recipients. Nephrol Dial Transplant;24(3):1051-3.

Westrell T, Teunis P, van den Berg H, Lodder W, Ketelaars H, Stenström TA, de Roda Husman AM (2006). Short- and long-term variations of norovirus concentrations in the Meuse river during a 2-year study period. Water Res;40(14):2613-20.

Whitehead K, McCue KA (2009). Virucidal efficacy of disinfectant actives against feline calicivirus, a surrogate for norovirus, in a short contact time. Am J Infect Control; 38 (1): 26-30.

WHO website: http://www.who.int/en/

Wilks SA, Keevil CW (2006). Targeting species-specific low-affinity 16S rRNA binding sites by using peptide nucleic acids for detection of Legionellae in biofilms. Appl Environ Microbiol;72(8):5453-62.

Wilson DA, Joyce MJ, Hall LS, Reller LB, Roberts GD, Hall GS, Alexander BD, Procop GW (2005). Multicenter evaluation of a Candida albicans peptide nucleic acid fluorescent in situ hybridization probe for characterization of yeast isolates from blood cultures. J Clin Microbiol;43(6):2909-12.

Winssinger N, Harris JL, Backes BJ, Schultz PG (2001). From split-pool libraries to spatially addressable microarrays and its application to functional proteomic profiling. *Angew Chem, Int Ed Engl*;40(17): 3152-3155.

Winssinger N, Ficarro S, Schultz PG, Harris JL (2002). Profiling protein function with small molecule microarrays. Proc Natl Acad Sci U S A;99(17):11139-44.

Wirgart BZ, Andersson P, Grillner L (2005). Evaluation of the ReSSQ assay in relation to the COBAS AMPLICOR CMV MONITOR test and an in-house nested PCR method for detection of cytomegalovirus DNA. J Clin Microbiol;43(8):4057-63.

Wojciechowski F, Hudson RH (2009). Peptide nucleic acid containing a meta-substituted phenylpyrrolocytosine exhibits a fluorescence response and increased binding affinity toward RNA. Org Lett;11(21):4878-81.

Wolf S, Williamson WM, Hewitt J, Rivera-Aban M, Lin S, Ball A, Scholes P, Greening GE (2007). Sensitive multiplex real-time reverse transcription-PCR assay for the detection of human and animal noroviruses in clinical and environmental samples. Appl Environ Microbiol;73(17):5464-70.

Wolffs P, Knutsson R, Sjöback R, Rådström P (2001). PNA-based light-up probes for real-time detection of sequence-specific PCR products. Biotechniques;31(4):766, 769-71.

Won BY, Yoon HC, Park HG (2008). Enzyme-catalyzed signal amplification for electrochemical DNA detection with a PNA-modified electrode. Analyst;133(1):100-4.

Wyn-Jones P (2007), "The Detection of Waterborne Viruses". Human Viruses in Water. Ed. Albert Bosch. New York: Elsevier Science Publishing Company, 2007. 177-204.

Xi C, Raskin L, Boppart SA (2005). Evaluation of microfluidic biosensor development using microscopic analysis of molecular beacon hybridization kinetics. Biomed Microdevices;7(1):7-12.

Xi JN, Graham DY, Wang KN, Estes MK (1990). Norwalk virus genome cloning and characterization. Science;250(4987):1580-3.

Yao C, Zhu T, Tang J, Wu R, Chen Q, Chen M, Zhang B, Huang J, Fu W (2008). Hybridization assay of hepatitis B virus by QCM peptide nucleic acid biosensor. Biosens Bioelectron;23(6):879-85.

Yin H, Lu Q, Wood M (2008). Effective exon skipping and restoration of dystrophin expression by peptide nucleic acid antisense oligonucleotides in mdx mice. Mol Ther;16(1):38-45.

Yoda T, Suzuki Y, Yamazaki K, Sakon N, Kanki M, Kase T, Takahashi K, Inoue K (2009). Application of a modified loop-mediated isothermal amplification kit for detecting Norovirus genogroups I and II. J Med Virol;81(12):2072-8.

Yoo JS, Kim CM, Kim JH, Kim JY, Oh JW (2009). Inhibition of Japanese encephalitis virus replication by peptide nucleic acids targeting cis-acting elements on the plus- and minus-strands of viral RNA. Antiviral Res;82(3):122-33.

Yoo SM, Choi JH, Lee SY, Yoo NC (2009). Applications of DNA microarray in disease diagnostics. J Microbiol Biotechnol;19(7):635-46.

Zelder FH, Mokhir AA, Krämer R (2003). Sequence selective hydrolysis of linear DNA using conjugates of Zr(IV) complexes and peptide nucleic acids. Inorg Chem;42(26):8618-20.

Zeng F, Peritz T, Kannanayakal TJ, Kilk K, Eiríksdóttir E, Langel U, Eberwine J (2006). A protocol for PAIR: PNA-assisted identification of RNA binding proteins in living cells. Nat Protoc;1(2):920-7.

Zerbi P, Schønau A, Bonetto S, Gori A, Costanzi G, Duca P, Vago L (2001). Amplified in situ hybridization with peptide nucleic acid probes for differentiation of Mycobacterium tuberculosis complex and nontuberculous Mycobacterium species on formalin-fixed, paraffin-embedded archival biopsy and autopsy samples. Am J Clin Pathol;116(5):770-5.

Zhang GJ, Chua JH, Chee RE, Agarwal A, Wong SM (2009). Label-free direct detection of MiRNAs with silicon nanowire biosensors. Biosens Bioelectron;24(8):2504-8.

Zhang GJ, Chua JH, Chee RE, Agarwal A, Wong SM, Buddharaju KD, Balasubramanian N (2008a). Highly sensitive measurements of PNA-DNA hybridization using oxide-etched silicon nanowire biosensors. Biosens Bioelectron;23(11):1701-7.

Zhang GJ, Zhang G, Chua JH, Chee RE, Wong EH, Agarwal A, Buddharaju KD, Singh N, Gao Z, Balasubramanian N (2008b). DNA sensing by silicon nanowire: charge layer distance dependence. Nano Lett;8(4):1066-70.

Zhao W, Ali MM, Brook MA, Li Y (2008). Rolling circle amplification: applications in nanotechnology and biodetection with functional nucleic acids. Angew Chem Int Ed Engl.;47(34):6330-7..

Zheng DP, Ando T, Fankhauser RL, Beard RS, Glass RI, Monroe SS (2006). Norovirus classification and proposed strain nomenclature. Virology;346(2):312-23.

Zielinski J, Kilk K, Peritz T, Kannanayakal T, Miyashiro KY, Eiríksdóttir E, Jochems J, Langel U, Eberwine J (2006). In vivo identification of ribonucleoprotein-RNA interactions. Proc Natl Acad Sci U S A;103(5):1557-62.

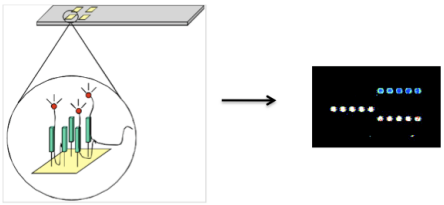
Aim of the work

An increasing number of food related issues is currently playing a key role in boosting new science investigations and new technological applications. From one side, molecular approaches help to assure food safety, quality and authenticity and arise new scientific questions in the field; on the other hand, a deeper knowledge of genomes helps to reveal essential information that allows to correlate genetics, medicine, nutrition and diagnostics. Chemistry and molecular biology often create and provide fundamental tools for these improvements.

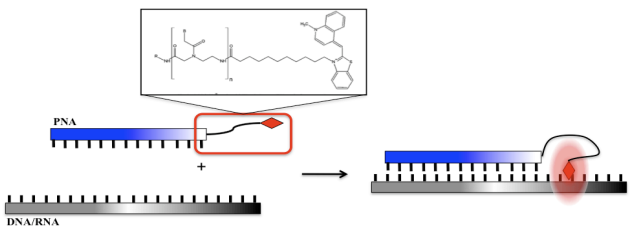
In this work, PNA-based systems to be used as tools for food safety and molecular biology are investigated and applications in diagnostics are envisaged as the relevant general aim. In particular, the following applications were developed:

A. New approaches for norovirus diagnostics: since norovirus represents an emerging food safety issue and, on the other hand, a good model system for RNA targeting studies, we focused our attention on the development of:

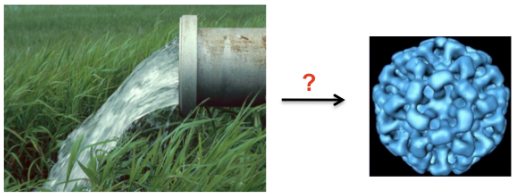
1.PNA-microarrays for norovirus detection: by combining the outstanding hybridization properties of PNAs for nucleic acid, with the microarray technology, we investigate the possibility to build a microarray platform with PNA probes, targeting short and highly conserved virus sequences (Chapter 2).



2. Thiazole orange (TO) conjugated PNAs for norovirus detection using isothermal based assays: the asymmetric cyanine dye thiazole orange is used to synthesize norovirus specific PNAs. The applicability of these probes in innovative diagnostic approaches such as the isothermal-based assays is investigated (Chapter 3)

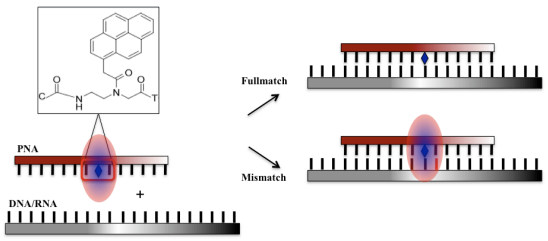


3. Tools for norovirus detection in water: the current methods for concentration of viruses form water are ususally cumbersome, expensive and time consuming. New approaches based on inexpensive materials such as glass wool fiber is investigated as a tool for the development of a filtering device for norovirus concentration and detection from water (Chapter 4).

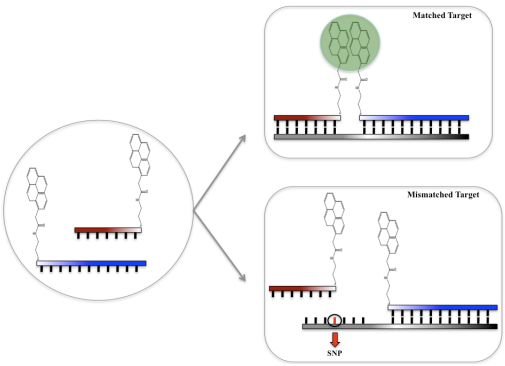


B. PNA-based strategies for sequence and point mutation analysis: since the development of effective methods for sequence analysis and point mutation detection is a key issue in diagnostics, two strategies involving derivatized PNA probes were investigated with the aim to develop:

1. A pyrenyl-PNA probe for DNA and RNA recognition: pyrene is a well known fluorophore employed in oligonucleotide labelling and derivatization. An approach based on a PNA probe bearing a pyrene moiety replacing a conventional nucleobase is investigated for DNA and RNA recognition and for mismatch recognition capabilities in a GC-rich sequence (Chapter 5).



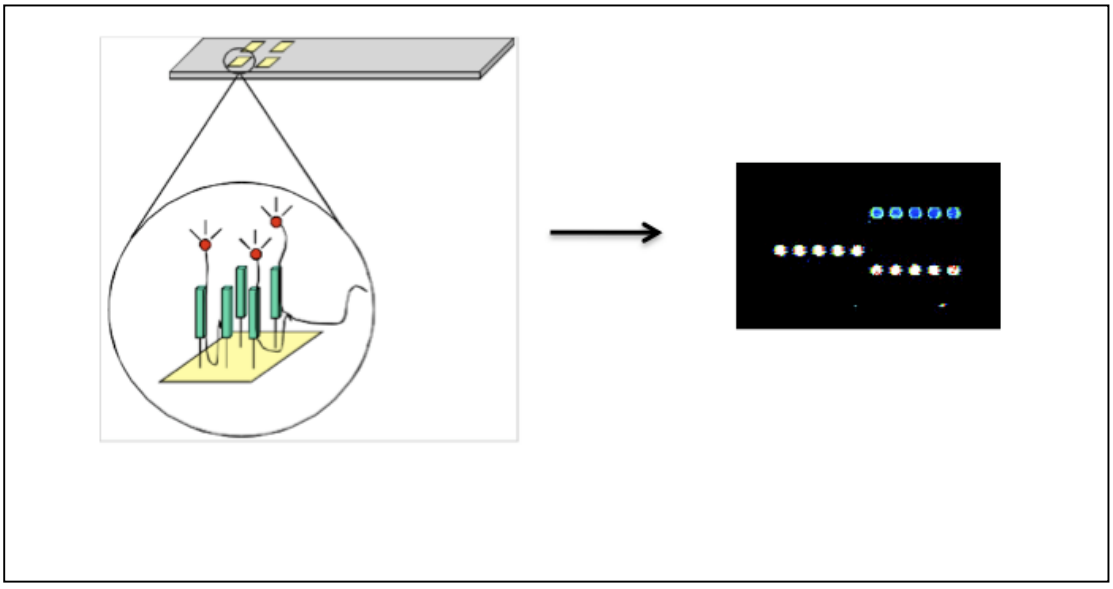
2. A dual-probe system based on pyrene-conjugated PNAs for point mutation detection: the employment of dual-probe systems for single nucleotide polymorphism analysis has been showed to be a valuable diagnostic tool. The development of two pyrene-conjugated PNA probes forming excimers upon hybridization with full match target is investigated as strategy to detect the PTPN22 C1858T polymorphism, a mutation involved in type 1 diabetes and other human autoimmune diseases (Chapter 6)



Chapter 2

Development of PNA-microarrays for Norovirus detection

A PNA microarray for the simultaneous detection of Norovirus (NoV) (GI and GII) genogroups has been developed, characterized and tested with stool specimens previously tested as positive for NoVs. The PNA probes were designed in order to target the most conserved stretches of nucleotides in the NoV genome and a single-tube, asymmetric, duplex RT-PCR was developed and optimized.



2.1 INTRODUCTION

Noroviruses (NoVs) are currently considered the most common cause of non-bacterial intestinal disease worldwide, accounting for more than 90% of viral gastroenteritis and approximately half of all outbreaks worldwide (Patel MM et al., 2009). NoV infection often occours by ingestion of contaminated food and water and, as major cause of secondary spread, from person-to-person transmission in enclosed settings such as cruise ships, hospitals, nursing homes and schools.

NoVs belong to the *Caliciviridae* family along with Sapovirus, the other calicivirus affecting humans, Lagovirus and Vesivirus which are related to animal infections (Green KY et al., 2000; Mayo MA, 2002).

The NoV genome is a single-stranded, positive-sense RNA of approximately 7.5 kb, organized in three open reading frames (ORF1-3). The currently proposed NoV classifications rely on sequence similarity evaluations, dividing NoVs in five genogroups (GI- to IV) which are further organized in a variable numbers of genotypes, according to the used similarity criteria (Zheng DP et al.,2006; Wang QH et al.,2007; Patel MM et al.,2009); however no official standard classification rules have been so far enstablished.

The lack of effective methods to propagate NoVs in cell culture is the main reason that forced researchers in focusing on molecular methods for NoV diagnostics both in clinical and environmental settings. The first established methods for NoV diagnostics were based on the electron microscopy (EM) and immunoelectron microscopy (IEM) (Atmar RL and Estes, 2001). However, these methods are cumbersome, relatively insensitive and require a high-level expertise for viral particle identification. The complete NoV genome sequencing (Jiang X et al., 1993; Lambden PR et al.,1993) made the devolpment of molecular-based methods possible for NoV analysis. In the last decade, several reverse transcription-PCR (RT-PCR) methods have been developed (Jiang XP et al., 1999; ;Ando T et al., 1995 Green J et al., 1993) and, in order to improve the test sensitivity, approaches based on nested reverse transcription-PCR (nRT-PCR) have also been proposed both for clinical (Medici MM et al., 2003) and food samples (Häfliger D et al.,1997). Currently, Real Time RT-PCR is considered as the gold-standard for NoV detection (Stals A et al., 2009; Wolf S et al., 2007; Jothikumar N et al., 2005; Pang XL et al., 2005). These methods allow to reduce the overall analysis time, to increase the sensitivity, to reduce the risks of cross-contamination and to simplify the laboratory operations.

Other molecular based techniques such as the isothermal amplification technologies like nucleic acid sequence-based amplification (NASBA) (Fukuda S et al., 2008; Lamhoujeb S et al., 2008; Kou X et al., 2006; Rutjes SA et al., 2006; Patterson SS et al., 2006; Moore C et al., 2004;Jean J et al., 2004; Greene SR et al., 2003) or loop-mediated isothermal amplification (LAMP) (Yoda T et al, 2009; Iturriza-Gómara M et al., 2008) have been proposed as effective alternative to the PCR-based methods.

Recently, interesting DNA microarray-based approaches including NoVs as targets have been published. A tiling microarray bearing 13,000 elements for the specific detection and characterization of common foodborne viruses, including NoVs, has been recently developed (Ayodeji M et al., 2009). A generic tag array to detect NoVs in water has also been developed to overcome the difficulties in environmental samples sequencing due to co-amplification of non-specific cDNAs (Brinkman NE and Fout GS, 2009). A microarray based on the hybridization of long PCR products (917 bp), that encompasses two major regions currently employed for the analysis of NoV genome, has been proposed as strategy to solve problems coming from the post PCR sequencing: short amplified products, often targeting different sequences, are difficult to compare and sometimes ambiguous for virus tracking in different research laboratories (Pagotto F et al, 2008). NoVs and Astroviruses have been also detected and genotyped in fecal samples using a microarray-based strategy (Jääskeläinen AJ and Maunula L, 2006). These approaches, however, are more focused on genotyping informations, in order to increase the throughput capability for screening purposes and rely on quite elaborate and expensive procedures.

The high polymophism of NoVs is still the major obstacle that limits the design of effective primers and probes to short regions within the most conserved part of the genome. Moreover, using non-chemically modified DNA probes, the needs for the identification of a sufficiently long stretch of conserved nucleotides is essential to assure an effective annealing in the temperature range commonly employed during the PCR based assays. A valuable strategy to overcome these limitations and to extend the selection of extremely short but highly conserved genomic regions, suitable for diagnostic purposes, might be represented by the introduction of modified probes or nucleic acid analogs, such as the Peptide Nucleic Acids (PNAs).

PNAs are oligonucleotide analogues in which the negatively charged sugar-phosphate backbone has been replaced by a chain of N-aminoethylglycine monomers (Nielsen PE, 1999). The high specificity and the higher affinity (thermal stability) for DNA and RNA compared with conventional DNA probes (Jensen KK et al., 1997; Dueholm KL et al., 1997),

make PNAs ideal tools for specifically targeting short sequences. They have been proposed as probes in microarray format in order to improve the efficiency and the stability of the assays (Weiler J et al., 1997). PNA microarrays have been successfully developed for the detection of Genetically Modified Organisms (GMOs) (Germini A et al., 2004; Germini A et al., 2005) and hidden allergens (Rossi S et al., 2006) in food stuffs. However, only one application of this technique has been recently reported for virus detection (Choi JJ et al., 2009).
In this work, the applicability of a PNA microarray for NoV detection and genogroup differentiation has been investigated. Two PNA probes, one specific for NoV GI and one specific for NoV GII were designed in order to maximize the detection events and used to fabricate a microarray which was characterized with complementary oligonucleotides and clinical samples.

2.2 MATERIALS AND METHODS

Sample preparation: i) Clinical specimens. The clinical specimens used for the microarray tests belong to the viral strain collection of the Department of Pathology and Laboratory Medicine of the University of Parma. The panel of positive stool samples, previously tested and characterized (Medici MC et al., 2005), include the following genogroups and genotypes: Desert Shield (GI), Hawaii (GII.1), Melksham (GII.2), Bristol (GII.4), Leeds (GII.7) and GII.b (GII. not assigned) . **ii) RNA extraction.** Viral RNA was extracted as previously described (Medici MC et al., 2005). Briefly, 250 μl of stool suspension was treated using the EXTRAgen kit (Nanogen Advanced Diagnostc S.r.l) according to the manufacturer's instructions. The RNA was resuspended in a final volume of 20 μl using nuclease-free water **iii) PCR amplification.** Asymmetric PCR amplifications were performed in a final volume of 50 μl, using a PCR System 9700 thermal cycler (Perkin Elmer Applied Biosystems) starting from 10 μl of extracted RNA. The single-tube RT-PCR was performed using the "SuperScriptTM One-Step RT-PCR for Long Templates" (Invitrogen, California, USA), and 0.1 μM (not-labelled) and 1.0 μM (Cy5-labelled) for primer concentrations. The PCR program were as follow: i) RT step: 58 °C for 30 min ii) amplification step: initial denaturation at 94 °C for 2 min followed by 40 cycles as follow: 94 °C for 30 sec, 58 °C for 1 min and 68°C for 1 min; and a final extension step at 68 °C for 5 min.

Primer and Probe design: The previously reported most conserved region in the norovirus genome (Kageyama T et al., 2003; Mohamed N et al., 2006) was used for probes design. All the available sequences were retrieved from GeneBank and aligned using BLAST. Conserved stretches of a minimun length of 12 nucleotides were identified and used to design specific probes fulfilling the following basic requirement: i) target one of the most conserved, specific sequence in the selected region of the genome; ii) obey to the general guidelines for sequence design of PNA oligomers; iii) identify the targeted region close to regions upstream and downstream suitable for primers definition. All the different sequences found and their respective frequences were taken into account in the probe design.

The same approach was applied for the primer design. After BLAST alignment, all the retrived sequences were used to identify all the different variants reported in the aligned regions and their respective frequences. Primers were defined according to the following criteria: i) minimize the Melting temperature (T_m)differences in order to set up a duplex PCR; ii) avoid intramolecular and primer-primer and primer-PNA probes interactions. The primers efficiencies and the theoretical T_m were evaluated using the Fast PCR software (Kalendar R et al., 2009).

PNA synthesis: The PNA oligomers were synthesized as described elsewhere (Lesignoli E et al., 2001). Briefly, PNAs were obtained by solid phase synthesis using a 433A Peptide Synthesizer (Applied Biosystem) following the synthetic BOC strategy. The addition of two spacer units of 2-(2-aminoethoxy)ethoxyacetic acid (AEEA) at the N-terminus of the PNAs was included in the automatized process. Swelling, downloading and cleavage of PNAs from the resin were performed manually. The probes were purified by reversed phase High-Performance Liquid Chromatography using a Phenomenex C18 peptide column (3 μm, 250 mm × 10 mm) with a binary gradient (flow rate: 4 ml/min) employing the following eluents: eluent A: water/TFA = 100:1; eluent B = water/acetonitrile/TFA = 100/40/1. The purified products were characterized by electrospray inonization mass spectrometry (Micromass ZMD). PNA_NoV GI: MS (ESI) estimated mass: 4014; measured mass: 4013; PNA_NoV GII: MS (ESI) estimated mass: 3457; measured mass: 3456.

Melting Temperature Measurements: PNAs dissolved in doubly distilled water were quantified using the following ε_{260} (M-1 cm-1) for the nucleobases: T=8,800; C=7,300; A=10,400; G=11,700. Melting temperature (T_m) measurements were determined using a Perkin Elmer Lamda Bio 20 UV/Vis spectrometer and quartz cuvettes. All the experiments

were carried out in buffered solution (100 mM NaCl, 10 mM NaH_2PO_4 pH 7.0) at a 5 μM concentration and 1 ml as final volume. Samples were heated to 90 °C and cooled at room temperature for 15 min. The melting curves were acquired between 18°C and 90°C with a temperature gradient of 1°C min $^{-1}$. The T_m of each sample was calculated from the maximum of the first derivative of the corresponding melting curve.

Array preparation: The amino terminal group of the PNA probes, was linked to the reactive N-hydroxysuccinimide ester groups on the solid support "CodeLink Activated Slides" (Amersham Bioscience). Deposition were performed using a SpotArray 24 (PerkinElmer) microarray printing system at room tempertature and controlled humidity. The spotting mixture was prepared with the appropriate probe concentration (10, 20 or 30 μM) opportunely diluted in a carbonate buffer (100mM, pH 9.0), containing 0.001% of sodium dodecyl sulfate (SDS). In order to avoid dragging of the probes and consequent contamination events in the subsequent depositions, the pins were washed three times after each probe deposition using a carbonate buffer (100mM, pH 9.0) additioned with different SDS concentrations: 0,2%, 1.0% and 0.001% respectively. The remaining reactive sites present on the slides surface after the probe deposition were blocked by overnight incubation in a humid chamber (relative humidity 75% at) at room temperature, followed by immersion for 30 min in a 50mM ethanolamine solution, 0.1mM TRIS, pH 9.0 prewarmed at 50 °C. The slides were washed twice with bidistilled water and then with a pre-warmed (50 °C) 4X saline-sodium citrate (SSC), 0.1% SDS solution under slow agitation. The slides were then washed with bidistilled water and the water was removed by centrifugation at 1500 rpm for 3 minutes.

Several sub-arrays with the same spotting layout were printed on different localization of the slides' surface and used for different analysis. The coordinates used for sub-array spotting were calculated in order to fit with the multi-well hybridization chamber Microarray Hybridization Cassette 1x16 (TeleChem International, Inc). The multi-well hybridization chamber was washed after each use with ethanol and rinsed thoroughly with doubly distilled water. The removable multiple-well silicon gasket was stored in doubly distilled water with 0.1% SDS and treated using the same procedure before and after each use.

In order to monitor the deposition quality, a fluorescent control probe (NH_2-$(AC)_{11}$-Cy5) was deposited in addiction to the PNA probes; all the washing operations were carried out protecting slides from direct light exposition in order to avoid degradation effects on the Cy5 fluorophore. The slides were stocked in dark plastic boxes until used.

Probes characterization: The performances of the PNA spotted probes were first evaluated by hybridizing mixtures of complementary Cy5 labelled synthetic DNA oligonucleotides at different concentrations. Each sample was hybridized in a different sub-array using the multiple hybridization chamber. The complementary oligonucleotides were diluted to the desired final concentration in a total volume of 65 μl using a 4X SSC, 0.1% SDS hybridization buffer. All the samples were hybridized at 45°C for 2 hours. After the hybridization step, each well containing the hybridization mixture was treated individualy without removing the silicon gasket as follow: the hybridization solution was removed by manual pipetting and 70 μl of 4X SSC, 0.1% washing solution prewarmed at 45 °C was added in each well and quickly removed after pipetting. A second aliquot of 70 μl of the same washing solution was than added and slides were washed for 5 minutes at 45°C under slow agitation. The washing solution was then removed from each well by manual pipetting and the slides were removed from the hybridization chamber and washed at room temperature with 0.2% SSC for 3 min and with 0.1% SSC for 1 min. The slides were then spin-dried for 3 min at 1500 rpm.

All the hybridization and washing procedures were carried out avoiding the slide exposition to direct light in order to prevent -Cy5 labelled samples degradation.

Sample Hybridization: The amplified, Cy5-labelled PCR fragments were prepared for microarray hybridization by dilution of 40 μl of the PCR reaction mixture to a final volume of 65 μl in the same hybridization buffer previously described and used with the complementary synthetic oligonucleotides. All the samples were hybridized at 40 °C for 2 hours and washed as previously indicated.

Image acquisition: Array immages were acquired using a ScanArray Express (PerkinElmer) at λex = 646 nm and λem = 664 nm with laser power = 100 and photomultiplier gain = 70

2.3 RESULTS AND DISCUSSION

2.3.1 Primer and probe definitions

In order to define a suitable combination of primers and probes for the amplification of NoVs GI and GII in duplex PCR format and for the subsequent hybridization using the PNA microarray, the highly conserved ORF1-ORF2 junction region (Kageyama T et al., 2003) was used to retrive all the complete and partial BLAST aligned genome sequences for both NoV genogroups. The main requirement during the sequence analythical process, was to identify the most conserved stretches of 12-15 nucleotides, suitable for the PNA probe design; moreover the potential probe region was selected taking into account the presence of conserved flanking regions enabling the design of primer pairs that allow to obtain short amplicons (max 100-130 bp), in order to maximize the microarray hybridization efficiency. One probe for each genogroup was defined and the sequence sub-populations bearing point mutations within the nucleotide stretch selected, along with the respective frequencies were estimated. The theoretical melting-temperature (T_m) of the two designed PNA probes was calculated and used to minimize differences between the two probes in order to optimize the hybridization process. According to the alignements of the retrived sequences (127 for GI and 795 for GII), the two designed probes are able to detect around 73.2% and 97.7% of the NoV strains belonging to the GI and GII respectively. The idea to use PNA probes instead of regular DNA probes, originate from the observation that very short stretches of 12-15 nucleotides are highly conserved along the NoV genome and that increasing the probe length up to 20 or more might increase the risk of finding mutated sequences, resulting in less efficient hybridization. This approach, aimed to shortening the target sequence, reminds of the efforts recently made to improve the Real Time PCR assays for norovirus, employing the locked nucleic acids (LNA) probes (Dreier J et al., 2006) or the minor groove binder (MGB) modified probes (Hymas W et al., 2007). However, we are aware about the extreme selectivity of the PNA probes, characteristic that might increase the risk of false negative even in the presence of a single point mutation. On the other hand, this absence of tolerance for the point mutations could be easily mitigated by the low number of probes required to cover 100% of the known NoV sequences and the number of PNA probes could be futhermore reduced designing chemically modified probes, which contain universal bases in place of regular nucleobases to compensate for the most represented point mutations. In order to evaluate the feasibility of a PNA platform to detect NoVs, one probe specific for each

genogroup was designed and synthesized for this study, according to the most represented sequences of GI and GII derived from the aligments.

The primers were defined with the major aim to be compatible with a duplex amplification assay (minimizing T_m differences) and the localization resulted similar to previously reported primers with minor modifications (Kageyama T et al., 2003; Hohne M and Schreier E, 2004; Jothikumar N et al., 2005; Mohamed N et al., 2006). However, the introduction of degenerate primer sequences was avoided and two different forward primers were designed and employed for GII in order to compensate the high polymorfism of the region.

Table 2.1. Primers and PNA probes selected in this study

Genogroup	Primer/Probe	Sequence (5' - 3')		Location[a]
GI	**NoV_GI_f**	**GCTGGATGCGCTTCCATGA**		5292 - 5310
	NoV_GI_r	**GCGTCCTTAGACGCCATCATC**		5360 -5380
	PNA_GI[(c)]	**CGATCTCCTGTCCA**	93/127 (73.2%)	5321 - 5334
	PNA_GI_v1	**T**GATCTCCTGTCCA	1/127 (0.8%)	
	PNA_GI_v2	CG**G**TCTCCTGTCCA	12/127 (9.4%)	
	PNA_GI_v3	CGATC**C**CCTGTCCA	13/127 (10.2%)	
	PNA_GI_v4	CGATCTCCTG**C**CCA	8/127 (6.3%)	
GII	**NoV_GII_fa**	**CAAGAGCCAATGTTCAGATGGATG**		5003 - 5026
	NoV_GII_fb	**CAAGAGCCAATGTTTAGGTGGATG**		5003 - 5026
	NoV_GII_r	**CGTCATTCGACGCCATCTTCA**		5086 - 5106
	PNA_GII[(c)]	**GATCGCCCTCCC**	777/795 (97.74%)[b]	5049 - 5060
	PNA_GII_v1	GATC**A**CCCTCCC	9/795 (1.13%)	
	PNA_GII_v2	GATC**C**CCCTCCC	3/795 (0.38%)	
	PNA_GII_v3	GATC**T**CCCTCCC	1/795 (0.12%)	
	PNA_GII_v4	GATCGCC**T**TCCC	4/795 (0.50%)	
	PNA_GII_v5	GATCGCC**G**TCCC	1/795 (0.12%)	

Notes: (a) Reference strains used: NoV GGI (accession no. M87661); NoV GGII (X87557); (b) numbers (and percentages) of sequences retrived from genebank matching the probe sequence; (c) sequences syntesized and used as probes in this study

The desinged primer pairs were then individually tested for their specificity using reference samples. The results were found to be consistent both for the amplicon length and for the primer specificity, assessed using reference material positive for related viruses, such as the rotavirus. The effect of the presence of five primers in the same PCR reaction was also evaluated and no aspecific amplifications were observed. PNA probes were synthesized by using a solid phase peptide synthesizer, following the protocol of the Boc synthesis. To the N-terminus of each oligomer two spacers AEEA were covalently linked in order to favour the accessibility of the probes to the microarray surface (Germini et al., 2005)

2.3.2 PNA microarray fabrication and characterization

The two PNA probes obtained (PNA_GI and PNA_GII) were first characterized in solution by determining the melting temperature with complementary oligonucleotides. For the PNA_GI and the PNA_GII probes the experimental Tm was 67.4 °C and 67.0 °C respectively.

A PNA microarray was obtained, as described in the experimental section, following protocols commonly used for oligonucleotides with the only exception of increasing washings with SDS between one probe spot and the other, in order to remove PNA tracks which can be sticked to the pin.

Afterwards in order to estimate the hybridization properties and the limit of detection of the developed microarray, the PNA probes were hybridized with Cy5-labelled synthetic oligonucleotides complementary to the probes, individually and simultaneously and at different concentrations (Figure 1). The two probes, spotted at a concentration of 30 μM, exhibited a similar behaviour: the hybridization performances resulted almost symmetrical; using a highly concentrated mix of both Cy5-labelled oligonucleotides (50 nM each), both the probes gave saturated signals whereas an unbalanced mix containing one of the two oligonucleotides at a concentration of 10 nM and the other at 90 nM, results in comparable signal intensities for both the PNA probes (Figure 2.1).

Moreover, the signal of the less concentrated oligonucleotide is the same when hybridized individually (Figure 2.1) confirming the absence of interference in the hybridization performances due to aspecific interactions between the PNA probes and the targeted sequences. The limit of detection resulted to be < 10 nM, since a good florescence enhancement was detected using a solution 10 nM of synthetic oligonucleotides, but no signals were detected down to 1 nM. This higher detection limit, compared to previous PNA microarray experiences (Germini A et al., 2004; Germini A et al. 2005; Rossi et al., 2006) might be due to the different sequence of the probes used in this work or to the slightly different washing process employed for the slides after the hybridization step.

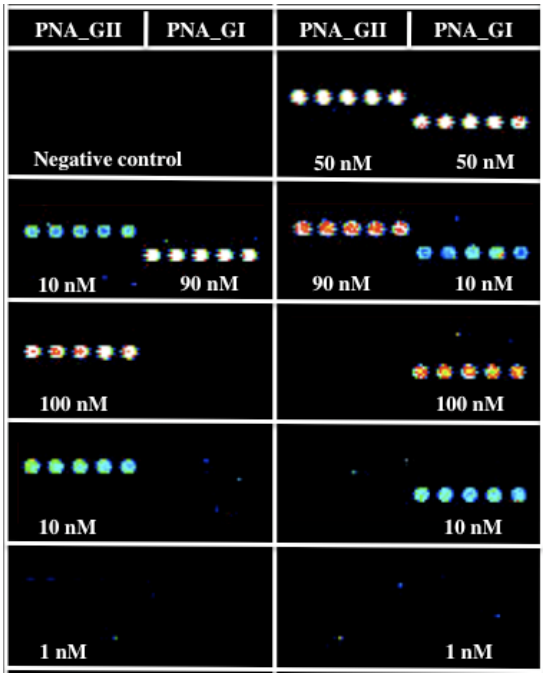


Figure 2.1. PNA microarray characterization: Each PNA probe was spotted five times. PNA_GI = spotted with NoGI_14mer; PNA_GII = spotted with NoGII_12merPNA. Different concentration of complementary Cy5-labeled oligonucleotides are individually or simultaneously hybridized onto the microarray. The concentrations for each olignonucleotide is indicated in each test. All samples were hybridized for 2 hours at 45°C

Since the microarray showed good performances using synthetic oligonucleotides, the system was tested using amplified samples with the attempt to evaluate the best PNA probe concentration. According to previuous experiences with the PNA microarray platforms, the best concentration of PNA probes seemed to be around 30 μM; however, all the previously reported evaluations were carried out using synthetic oligonucleotides in a buffered solution, mimicking the PCR amplicons; thus, all these characterizations did not take into account the variability and the more complex environment coming from the PCR reaction. In order to test the best probe concentration for real samples, amplified positive reference NoV GII specimen were hybridized in the presence of different concentrations of the PNA_GII probe (Figure 2.2). The results showed that when the hybridization is efficient (Figure 2.2a), a probe concentration around 30 μM give a high (close to the saturation) signal intensity; however, when the hybridization efficiency is low, no notable improvements come from an increased probe concentration. These results partially confirmed the previously reported observations (Germini A et al., 2004; Germini A et al. 2005; Rossi et al., 2006) and a concentration of 30 μM for both probes were considered optimal for the subsequent experiments.

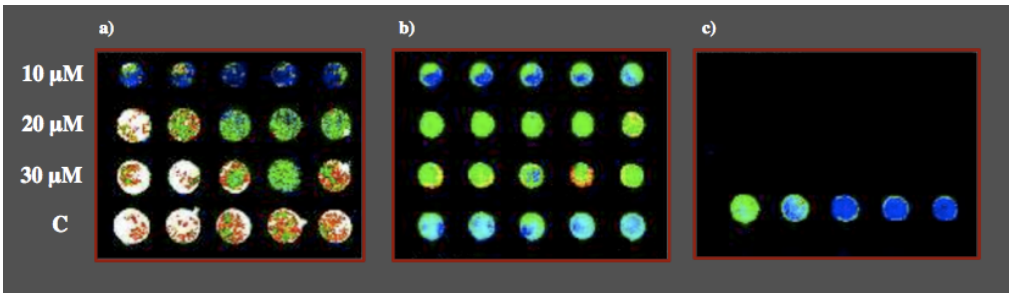


Figure 2.2. Evaluation of the probe concentration on signals intensity. PNA_GII probe hybridized with DNA amplified from two reference nRT-PCR positive samples (a, b) and a PCR negative control using extracted RNA from rotavirus (c). C = Deposition control

2.3.3 Detection of NoV specimens

The developed PNA microarray was tested with stool specimens collected at the Department of Pathology and Laboratory Medicine of the University of Parma during the years 2000-2004 and previously assessed as positive for NoVs by an internally developed nRT-PCR (Medici MC et al., 2003). All the samples coming from the same extraction session were analyzed at the same time using the PNA microarray and the nRT-PCR previously described as reference method. Several genotypes belonging to the genogroup II were selected among the available samples, in order to evaluate the applicability of the PNA microarray as broadly reactive screening method. Unfortunately, only one sample of norovirus belonging to the genogroup I was available during the experiments. Therefore it was not possible to perform an exhaustive evaluation for this genogroup.

Table 2.2. Detection of norovirus (different genotypes) by PNA-microarray and by nested PCR, as reference method. Each extracted sample was tested in parallel using the two methods

Genogroup/genotype		Nested PCR[a]	Microarray	Concordance (%)
GI:				
Desert Shield	1	0/1	0/1	100
GII:				
GII.4	5	5/5	4/5	80
Hawaii	3	3/3	3/3	100
Leeds	2	2/2	2/2	100
Melksham	1	0/1	0/1	100
Rotterdam	1	0/1	0/1	100
GII.b	13	10/13	10/13	100
Tot:	26			

Note: (a) Medici MM et al., 2003

The extracted nucleic acid from each sample underwent a single RT-PCR amplification, as described in the materials and methods section, using an excess of Cy5-labelled primer and was hybridized on the PNA microarray without any further treatment. The results are summarized in Table 2.2 and the relative microarray images are reported in Figure 2.3.

The PNA microarray showed concordant results with the nRT-PCR method taken as confirmatory reference method, except for a sample of the genotipe GII.4 (Figure 2.3, b-i). It is intresting to point out that some samples resulted negative using both techniques; however, it was not possible to define the reasons that led to this false negative. A possible explanation might be the effect of sample degradation: several samples were stored for long time and underwent several freeze-thaw cycles that potentially led to a massive genomic RNA damage. Moreover the scarce amount of some samples made not feasible a systematic screening in order to further optimize the conditions during the PNA microarray assay. The PNA microarray failed to detect the only available GI sample. However, the inability to detect the strain belonging to the Desert Shield genotype was paralleled by the result obtained with the nRT-PCR which was not efficient with this genotype (Medici MM et al., 2003).

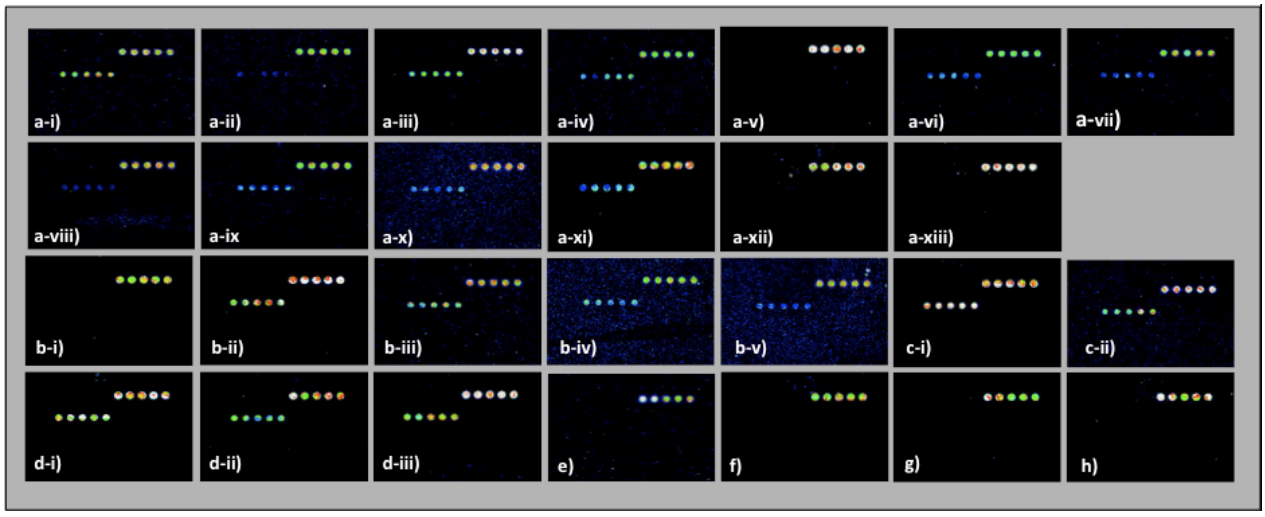


Figure 2.3. PNA microarray analyses of reference positive material (fecal suspensions) containing different norovirus genotypes. a-(i-xiii): GII.b (GII. not assigned); b-(i-v): GII.4 (Bristol); c-(i-ii): GII.7 (Leeds); d-(i-iii): GII.1 (Hawaii); e) non-target control; f) GII.2 (Melksham); g) GII.2 (Rotterdam)

The failed detection using the PNA microarray might be due to the more stable secondary structure formed by the GI amplicons (Figure 2.4) or to the presence of a mutation in the region targeted with the probe: according to the alignment previously described, the GI sequence seems to be more variable even in the highly conserved domains. Unfortunately no further investigations were possible for GI samples, since no other samples were available.

Another remarkable aspect concerning the PNA microarray is the good results obtained hybridizing the samples after the asymmetric PCR amplification without any furher processation. The previously reported PNA microarray platforms employed a combination of two PCR processes: a first conventional PCR followed by a second asymmetric PCR amplification (Germini A et al., 2005; Rossi S et al., 2006) or treatments post-PCR amplification with the λ-exonuclease (Germini A et al., 2004) in order to obtain a good amount of single strand DNA of the amplified targets. By the present method, the single tube asymmetric RT-PCR developed is sufficient to succesfully detect NoVs extracted from stool specimens. However, the detection limit for the whole method (extraction-detection-hybridation) was not investigated and consequently, the needs for enrichment strategies, such as the employment of two subsequent PCR amplification rounds, cannot be completely excluded for the less concentrated samples.

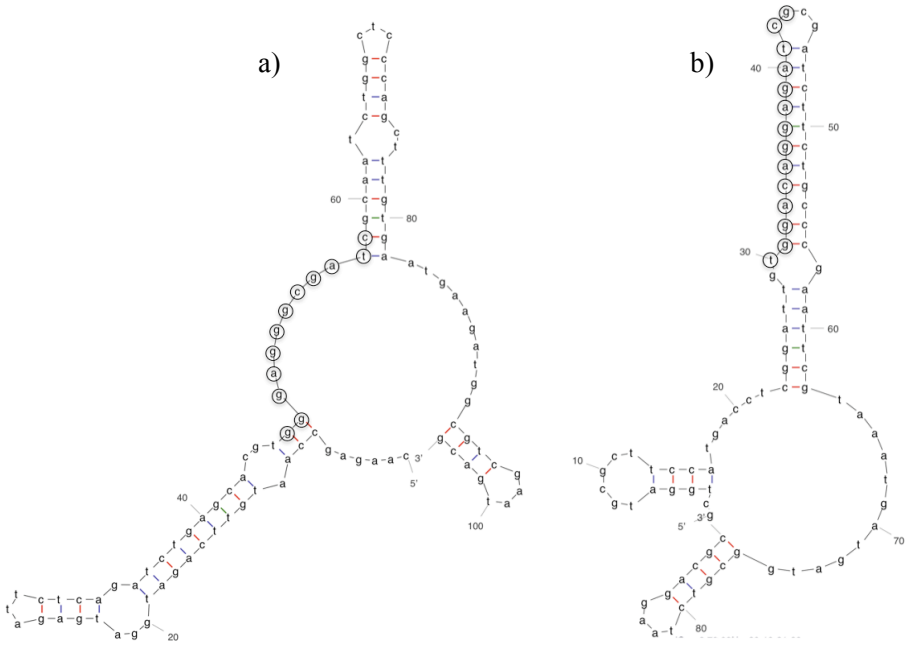


Figure 2.4. Evaluation of the secondary structures for the single-stranded GI (a) and GII (b) amplicons. The nucleobases targeted by the PNA probes are marked with black circles. The structures were calculated using mfold (Zuker M, 2003) with default parameters

Other microarray-based strategies were proposed with the aim to perform sample genotyping along with the virus detection in the same reaction. These brilliant approaches, however, rely on more complicated procedures and are more time demanding. The PNA microarray on the other hand, has the advantage to be easy and fast and the shorter probes based on PNA allow to detect the majority of the virus variants with a limited numbers of probes. Another noteworthy advantage of the microarray format is the fact that it is possible to separate the

PCR-related optimization procedures from the conditions required for an efficient amplicon hybridization to the PNA probes. This strategy could represent a more flexible aproach, particularly useful for targeting viruses with high sequence diversity, such as NoVs: the constant needs for primer improvements can be managed without taking into account the probe design phase since the amplicon hybridization does not occour during the PCR reaction. Conversely, the introduction of new probes into the array library will not affect the PCR optimization. Furthermore, the microarray platform easily allows the integration of other targets in the same assay leading to the construction of custom arrays. Moreover, the good performances of the PNAs as specific capture probes for NoV detection might enable the adaptation of this strategy to other platforms like sensors, microfluidic devices or systems for point-of-care analyses.

2.4 CONCLUSIONS

Two PNA probes for NoV detection and simultaneous genogroup identification were designed and synthesized. The probes sequence definition was based on the identification of short, higly conserved stretches of about 12-15 nucleotides in the ORF1-ORF2 junction region. PNAs give the possibility to rely on good specificity and high melting temperatures even for extremely short (10-15) oligomers length. This characteristic allowed a design approach difficult to achieve with standard DNA oligonucleotides, targeting shorter and more conserved regions of the NoV, minimizing the risks for sequence polymorphisms. According to the analyses on the BLAST aligned sequences retrived from public databases, these two PNA probes are able to detect with fullmatch precision aproximately the 73.2% and 97.7% of the sequences available for the GI and the GII genotypes respectively. The PNA probes were used to construct a microarray in order to evaluate the applicability of this technique for NoV diagnostics. NoVs samples belonging to different genotypes were extracted from stool specimens and analyzed using the PNA microarray; the same samples were evaluated using a previously developed nested PCR, as reference method. The results were in accordance for all the samples tested but one, belonging to the GII.4 genotype.

Moreover, the single-tube, asymmetric RT-PCR optimized for the assay, resulted effective for the direct hybridization of single-stranded amplicons, without requirement for other processation steps. This makes the PNA microarray an easy and fast assay for NoV detection and genogroup differentiation. The possibility to produce customized microarrays expanding the probe sets provides a very flexible approach. Furthermore, the good performaces observed

for the surface-bound PNA probes might open new possibilities for the employment of PNA-based strategies in other surfaces-based detection methods for NoV diagnostics.

2.5 REFERENCES

Ando T, Monroe SS, Gentsch JR, Jin Q, Lewis DC, Glass RI (1995). Detection and differentiation of antigenically distinct small round-structured viruses (Norwalk-like viruses) by reverse transcription-PCR and southern hybridization. J Clin Microbiol;33(1):64-71.

Atmar RL, Estes MK (2001). Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses. Clin Microbiol Rev;14(1):15-37.

Ayodeji M, Kulka M, Jackson SA, Patel I, Mammel M, Cebula TA, Goswami BB (2009). A microarray based approach for the identification of common foodborne viruses. Open Virol J;3:7-20.

Baert L, Uyttendaele M, Debevere J (2007). Evaluation of two viral extraction methods for the detection of human noroviruses in shellfish with conventional and real-time reverse transcriptase PCR. Lett Appl Microbiol;44(1):106-11.

Brinkman NE, Fout GS (2009). Development and evaluation of a generic tag array to detect and genotype noroviruses in water. J Virol Methods;156(1-2):8-18.

Choi JJ, Kim C, Park H (2009). Peptide nucleic acid-based array for detecting and genotyping human papillomaviruses. J Clin Microbiol;47(6):1785-90.

Dreier J, Störmer M, Mäde D, Burkhardt S, Kleesiek K (2006). Enhanced reverse transcription-PCR assay for detection of norovirus genogroup I. J Clin Microbiol;44(8):2714-20.

Dueholm KL and Nielsen PE (1997). Chemistry, properties and applications of PNA (peptide nucleic acid). New J. Chem.; *21*, 19-31.

Fukuda S, Takao S, Kuwayama M, Shimazu Y, Miyazaki K (2006). Rapid detection of norovirus from fecal specimens by real-time reverse transcription-loop-mediated isothermal amplification assay. J Clin Microbiol;44(4):1376-81.

Germini A, Rossi S, Zanetti A, Corradini R, Fogher C, Marchelli R. Development of a peptide nucleic acid array platform for the detection of genetically modified organisms in food. J Agric Food Chem. 2005 May 18;53(10):3958-62.

Germini A, Mezzelani A, Lesignoli F, Corradini R, Marchelli R, Bordoni R, Consolandi C, De Bellis G (2004). Detection of genetically modified soybean using peptide nucleic acids (PNAs) and microarray technology. J Agric Food Chem;52(14):4535-40.

Green J, Norcott JP, Lewis D, Arnold C, Brown DW (1993). Norwalk-like viruses: demonstration of genomic diversity by polymerase chain reaction. J Clin Microbiol;31(11):3007-12.

Green KY, Ando T, Balayan MS, Berke T, Clarke IN, Estes MK, Matson DO, Nakata S, Neill JD, Studdert MJ, Thiel HJ (2000). Taxonomy of the caliciviruses. J Infect Dis;181 Suppl 2:S322-30.

Greene SR, Moe CL, Jaykus LA, Cronin M, Grosso L, Aarle P (2003). Evaluation of the NucliSens Basic Kit assay for detection of Norwalk virus RNA in stool specimens. J Virol Methods;108(1):123-31.

Hymas W, Atkinson A, Stevenson J, Hillyard D (2007). Use of modified oligonucleotides to compensate for sequence polymorphisms in the real-time detection of norovirus. J Virol Methods;142(1-2):10-4.

Häfliger D, Gilgen M, Lüthy J, Hübner P (1997). Seminested RT-PCR systems for small round structured viruses and detection of enteric viruses in seafood. Int J Food Microbiol;37(1):27-36.

Hohne, M, Schreier, E (2004), Detection and characterization of norovirus outbreaks in Germany: application of a one-tube RT-PCR using a fluorogenic real-time detection system. J. Med. Virol; 72, 312–319

Iturriza-Gómara M, Xerry J, Gallimore CI, Dockery C, Gray J (2008). Evaluation of the Loopamp (loop-mediated isothermal amplification) kit for detecting Norovirus RNA in faecal samples. J Clin Virol;42(4):389-93.

Jean J, D'Souza DH, Jaykus LA (2004). Multiplex nucleic acid sequence-based amplification for simultaneous detection of several enteric viruses in model ready-to-eat foods. Appl Environ Microbiol;70(11):6603-10.

Jensen KK, Orum H, Nielsen PE, Nordén B (1997). Kinetics for hybridization of peptide nucleic acids (PNA) with DNA and RNA studied with the BIAcore technique. Biochemistry;36(16):5072-7.

Jiang X, Huang PW, Zhong WM, Farkas T, Cubitt DW, Matson DO (1999). Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR. J Virol Method;83(1-2):145-54.

Jiang X, Wang M, Wang K, Estes MK (1993). Sequence and genomic organization of Norwalk virus. Virology;195(1):51-61.

Jothikumar N, Lowther JA, Henshilwood K, Lees DN, Hill VR, Vinjé J (2005). Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. Appl Environ Microbiol;71(4):1870-5.

Jääskeläinen AJ, Maunula L (2006). Applicability of microarray technique for the detection of noro- and astroviruses. J Virol Methods;136(1-2):210-6.

Kageyama T, Kojima S, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Takeda N, Katayama K. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J Clin Microbiol. 2003 Apr;41(4):1548-57.

Kalendar R, Lee D, Schulman AH (2009). FastPCR Software for PCR Primer and Probe Design and Repeat Search. Genes, Genomes and Genomics; 3(1): 1-14.

Kou X, Wu Q, Zhang J, Fan H. Rapid detection of noroviruses in fecal samples and shellfish by nucleic acid sequence-based amplification. J Microbiol. 2006 Aug;44(4):403-8.

Lambden PR, Caul EO, Ashley CR, Clarke IN (1993). Sequence and genome organization of a human small round-structured (Norwalk-like) virus. Science;259(5094):516-9.

Lamhoujeb S, Fliss I, Ngazoa SE, Jean J (2008). Evaluation of the persistence of infectious human noroviruses on food surfaces by using real-time nucleic acid sequence-based amplification. Appl Environ Microbiol;74(11):3349-55.

Lesignoli E, Germini A, Corradini R, Sforza S, Galavema G, Dossena A, Marchelli R (2001). Recognition and strand displacement of DNA oligonucleotides by peptide nucleic acids (PNAs). High-performance ion-exchange chromatographic analysis. J Chromatogr A;922(1-2):177-85.

Mayo MA (2002). A summary of taxonomic changes recently approved by ICTV. Arch Virol;147(8):1655-63.

Mohamed N, Belák S, Hedlund KO, Blomberg J (2006). Experience from the development of a diagnostic single tube real-time PCR for human caliciviruses, Norovirus genogroups I and II. J Virol Methods;132(1-2):69-76..

Moore C, Clark EM, Gallimore CI, Corden SA, Gray JJ, Westmoreland D (2004). Evaluation of a broadly reactive nucleic acid sequence based amplification assay for the detection of noroviruses in faecal material. J Clin Virol;29(4):290-6.

Nielsen PE (1999). Peptide Nucleic Acid. A Molecule with Two Identities. Accounts of Chemical Research, 32: 624-630.

Pagotto F, Corneau N, Mattison K, Bidawid S (2008). Development of a DNA microarray for the simultaneous detection and genotyping of noroviruses. J Food Prot;71(7):1434-41.

Pang XL, Preiksaitis JK, Lee B (2005). Multiplex real time RT-PCR for the detection and quantitation of norovirus genogroups I and II in patients with acute gastroenteritis. J Clin Virol;33(2):168-71.

Patel MM, Hall AJ, Vinjé J, Parashar UD (2009). Noroviruses: a comprehensive review. J Clin Virol;44(1):1-8.

Patterson SS, Smith MW, Casper ET, Huffman D, Stark L, Fries D, Paul JH (2006). A nucleic acid sequence-based amplification assay for real-time detection of norovirus genogroup II. J Appl Microbiol;101(4):956-63.

Rossi S, Scaravelli E, Germini A, Corradini R, Fogher C, Marchelli R (2006). A PNA-array platform for the detection of hidden allergens in foodstuffs. European Food Research and Technology. Eur. Food Res. Technol., *223*, 1–6.

Rutjes SA, van den Berg HH, Lodder WJ, de Roda Husman AM (2006). Real-time detection of noroviruses in surface water by use of a broadly reactive nucleic acid sequence-based amplification assay. Appl Environ Microbiol;72(8):5349-58.

Stals A, Baert L, Botteldoorn N, Werbrouck H, Herman L, Uyttendaele M, Van Coillie E (2009). Multiplex real-time RT-PCR for simultaneous detection of GI/GII noroviruses and murine norovirus 1. J Virol Methods;161(2):247-53.

Wang QH, Costantini V, Saif LJ (2007). Porcine enteric caliciviruses: genetic and antigenic relatedness to human caliciviruses, diagnosis and epidemiology. Vaccine;25(30):5453-66.

Weiler J, Gausepohl H, Hauser N, Jensen ON, Hoheisel JD (1997). Hybridisation based DNA screening on peptide nucleic acid (PNA) oligomer arrays. Nucleic Acids Res;25(14):2792-9.

Wolf S, Williamson WM, Hewitt J, Rivera-Aban M, Lin S, Ball A, Scholes P, Greening GE (2007). Sensitive multiplex real-time reverse transcription-PCR assay for the detection of human and animal noroviruses in clinical and environmental samples. Appl Environ Microbiol;73(17):5464-70.

Yoda T, Suzuki Y, Yamazaki K, Sakon N, Kanki M, Kase T, Takahashi K, Inoue K (2009). Application of a modified loop-mediated isothermal amplification kit for detecting Norovirus genogroups I and II. J Med Virol;81(12):2072-8.

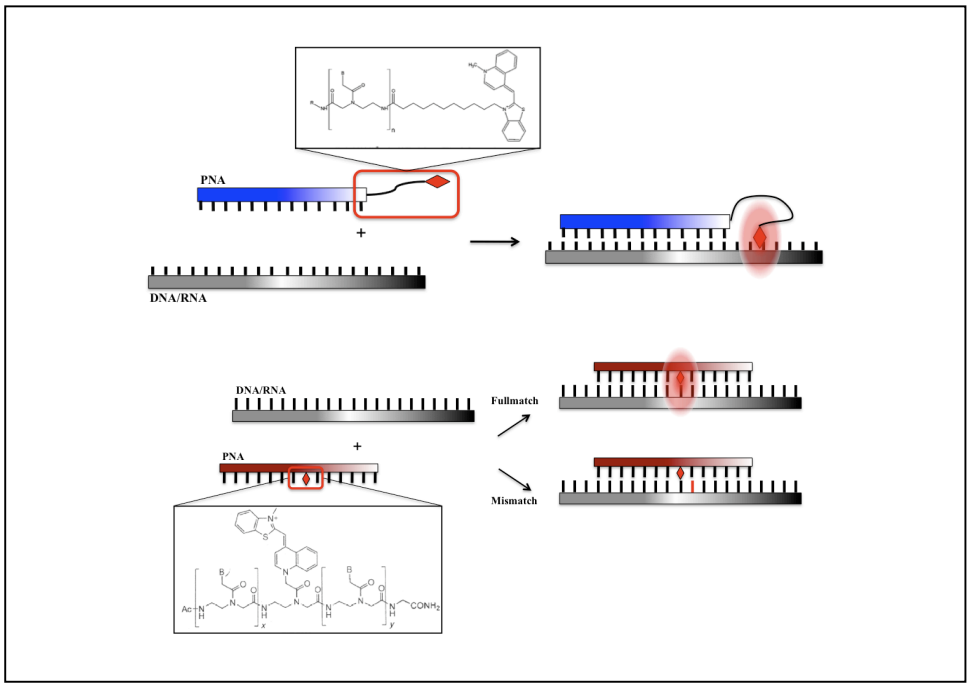
Zheng DP, Ando T, Fankhauser RL, Beard RS, Glass RI, Monroe SS (2006). Norovirus classification and proposed strain nomenclature. Virology;346(2):312-23.

Zuker M (2003). Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res;31(13):3406-15.

Chapter 3

PNAs conjugated with Thiazole Orange (TO) as Light-Up and FIT probes for norovirus detection

Thiazole orange (TO) conjugated peptide nucleic acid (PNA) probes for GII noroviruses (NoV) detection were designed and synthesized. The PNA-probes target the most conserved stretch of nucleotides located in a highly conserved portion of the NoV genome, the open reading frame 1-2 (ORF1-ORF2) junction region The two probes devised have analogous sequence but differ for the dye conjugation strategy: i) end-labelling, with the TO molecule tethered by a linker to the end of the PNA probe; ii) nucleobase substitution, with the TO molecule directly linked to the PNA backbone replacing a conventional nucleobase. The spectroscopic properties of the two PNA probes were studied and their applicability to NoVs detection, using an isothermal assay, was investigated. Both probes showed good specificity and high fluorescence enhancement upon hybridization, especially targeting RNA molecules. Moreover, the two probes were successfully employed for detection of NoVs from stool specimens in an isothermal-based amplification assay. The probes showed to be specific even in the presence of high concentrations of non-target RNA.



3.1 INTRODUCTION

The current demanding for rapid, sensitive and robust nucleic acid amplification tests (NAATs) is one of the most challenging task in the post-genomic era. Most of these methods are currently based on the detection of fluorescence changes occuring during the assay, as a consequence of the specific target amplification; these reporter signal detections are usually based on the interaction of the amplified nucleic acids with binding dyes or with oligonucleotide detection probes or primers labelled with an appropriate fluorophore that increases their fluorescence emission upon specific target sequence hybridization (Mackay J and Landt O, 2007).

The application of innovative approaches in probes development, is currently the subject of intense researches in synthetic chemistry and new probes, designed for particular detection purposes, are rapidly gaining ground in the NAATs arena. Promising results have been achieved tethering molecules to oligomers by means of proper linkers or with the insertion of fluorescent base analogues into the probe sequence. Numerous fluorophores and probes are already available for specific purposes (Ranasinghe RT and Brown T, 2005; Okamoto A et al., 2005; Venkatesan N et al., 2008).

The functionalization of Peptide Nucleic Acid (PNA) oligomers with the asymmetric cyanine dye Thiazole Orange (TO) was shown to be an effective strategy in nucleic acid analysis. The peculiar properties of this positively charged heterocycle, the strong fluorescence enhancement upon DNA binding and the well-known base staking ability, were sucessfully exploited in different PNA derivatization approaches:

TO-tethered PNA probes (the Light-Up approach)

In previous works, Kubista's group constructed the so called "Light-Up" probes: a TO molecule is covalently linked to the N-terminus of the PNA oligomers by a linker using two different binding configurations (Figure 3.1). This kind of probes has shown a weak fluorescence signal when they are alone but a high fluorescence enhancement upon hybridization. Therefore they are suitable for specific detection of nucleic acids in homogeneous assays (Svanvik N et al., 2000a). The same strategy was applied to detect post-PCR products such as the human b-actin gene and the *invA* gene of *Salmonella* with high resolution of a single mismatch. (Isacsson J et al., 2000). Moreover, Real Time detection assays for the *gusA* gene of the *Escherichia coli* ß-glucuronidase (Svanvik N et al., 2000b) and for part of the plasmid-borne virulence gene *yadA* (Wolffs P et al., 2001) have been

developed with Light-Up probes. These modified PNA oligomers have also been successfully applied in clinical diagnostic applications (Wirgart BZ et al., 2005; Leijon M et al., 2006) and the same technology has been implemented in commercial kits under the name of ReSSQ® line (Wirgart BZ et al., 2005).

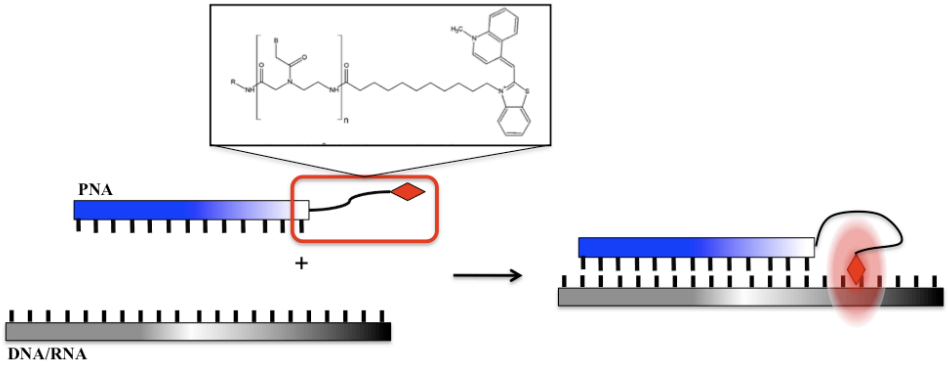


Figure 3.1. Schematic representation of the Light-Up probes

TO as base surrogate in PNA probes (FIT approach)

The substitution of an internal nucleobase by an intercalator was investigated by Seitz's group as a tool to obtain probes responsive to structural perturbations and local changes generated by single base mismatches in target DNAs upon hybridization. PNA probes containing quinoline linked TO as universal base, have shown to be responsive to alterations induced by mismatches (Köhler O and Seitz O, 2003). This kind of probes, named Forced Intercalation Probes (FIT) by the inventors, has been extensively studied for SNPs detection purposes. (Köhler O et al., 2005; Bethge L et al., 2008). Recently FIT probes, along with an improved version bearing a D-ornithine rather than aminoethylglycine in the PNA backbone, have been succesfully used in a Real Time PCR assay to detect two SNPs in the gene encoding for the cystic fibrosis transmembrane conductance regulator (CFTR) protein. (Socher E at al., 2008)

Despite these TO-based approaches have revealed encouraging performances both in model systems and in DNA detection in several PCR formats, no reports targeting RNA instead of DNA products have been published so far. PCR-based methods are currently recognised as golden standards in nucleic acid detection, however, isothermal amplification technologies such as Nucleic Acid Sequence-Based Amplification (NASBA) (Deiman B et al., 2002), Loop-Mediated Isothermal Amplification (LAMP) (Parida M et al., 2008, Mori Y and Notomi

T, 2009) or Rolling Circle Amplification (RCA) (Zhao W et al., 2008) are increasingly gaining popularity as effective alternatives for specific purposes. These kind of assays, usually based on a combination of different enzymes, do not require thermal cycling procedures, thus allowing to perform the target amplification step under isothermal conditions. Isothermal amplification assays are particularly suitable in clinical and environmental applications, for specific tasks such as viral RNA genome detection or bacterial viability evaluation (Keer JT and Birch L, 2003; Weile J and Knabbe C, 2009).

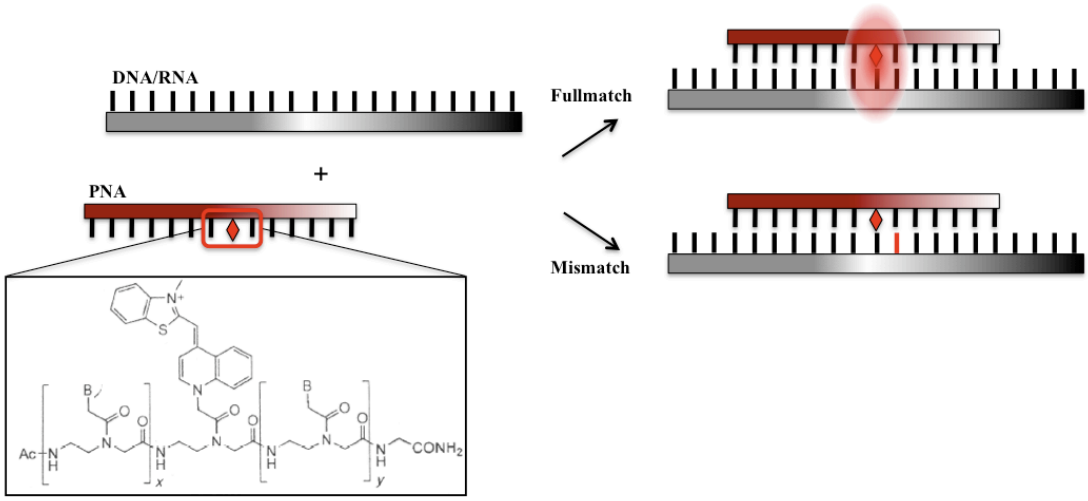


Figure 3.2. Schematic representation of the FIT probes

In the present work, the applicability of the TO-derivatized PNA probes to Real Time RNA-target transcription monitoring is investigated. The main aims are: i) to design a PNA probe for the most conserved region of the norovirus GII genome, a single stranded RNA, nonenveloped virus which is supposed to be the most common agent of non bacterial gastroenteritis worldwide (Patel MM et al., 2009); ii) to synthesize, characterize and evaluate the performance of Light-Up- and FIT- like probes for the chosen sequence; iii) to evaluate the behaviour of the probes in an isothermal amplification model system targeting NoV amplified nucleic acid from clinical samples.

3.2 MATERIALS AND METHODS

Probe design: The previously reported Norovirus GII most conserved region (Kageyama et al., 2003) was used for probes design. All the available sequences were retrieved from GeneBank and aligned using BLAST. Conserved sequences of 12 nucleotides as the minimum length were identified using CLC Sequence viewer 6 (Aarhus, Denmark) as graphical tool. These fragments were used to define a probe sequence which fulfills the following requirements: i) target a highly conserved, specific sequence; ii) obey to the general guidelines for PNA oligomer sequence design; iii) allow the identification of conserved regions upstream and downstream for primer definition.

TO-PNA probes synthesis and characterization: PNAs were synthesized by using an ABI 433A authomatic synthesizer, using commercially avaible PNA monomers (Applied Biosystems) and following Boc synthesis protocols for standard PNA solid phase synthesis. The Thiazole orange units, 6-(thiazole orange) exanoic acid and 2-(thiazole orange) ethanoic acid were coupled to the PNA backbone by using DIC/DhBTOH manual standard coupling procedure. PNAs were purified by semipreparative HPLC (final purity >80%) and characterized by ESI-MS.

LightUp_NoVGII, ESI-MS (H_2O positive ions): m/z: 748 $[MH_4]^{4+}$, 598 $[MH_5]^{5+}$, 499 $[MH_6]^{6+}$.

FIT_NoVGII, ESI-MS (H_2O positive ions): m/z: 927 $[MH_3]^{3+}$, 695 $[MH_4]^{4+}$, 556 $[MH_5]^{5+}$, 464 $[MH_6]^{6+}$.

Melting temperature measurements: Melting temperature (T_m) measurements were determined using a Perkin Elmer Lamda Bio 20 UV/Vis spectrometer and quartz cuvettes. All the experiments were carried out in a buffered solution (100 mM NaCl, 10 mM NaH_2PO_4 pH 7.0) at a 5 μM concentration and 1 ml as final volume. Samples were heated to 90 °C and cooled at room temperature for 15 min. The melting curves were acquired between 18°C and 90°C with a temperature gradient of 1°C min^{-1}; the T_m of each sample was calculated from the maximum of the first derivative of the corresponding melting curves.

Fluorescence mesurements: i) Studies of complexation of the TO-modified probes with synthetic oligonucleotides were carried out in buffered solution (100 mM NaCl, 10 mM NaH_2PO_4 pH 7.0) using fluorescence quartz cuvettes and a final volume of 100 μl. If no

different concentrations are specified, all the mesurement are acquired at 1 μM, with a Perkin Elmer LS 55 spectrofluorimeter using 470 nm as excitation wavelength, with $slit_{Ex}$ = 2.5 and $slit_{Em}$ = 10.0.

The monitoring of RNA trnscription was performed with a Perkin Elmer Victor 3 spectrofluorometer selecting the fluorescein filters, in a 384-well fluorescence plate in a final volume of 20 μl. Five scans were taken and averaged for each data point.

Polymerase chain reaction (PCR) and T7 polymerase-based isothermal amplification:

A two-step amplification-transcription system, based on PCR amplification followed by a subsequent T7 polymerase specific transcription, was built up to evaluate the TO conjugated PNA probes.

Samples of NoV and Rotavirus (RV) were extracted from stool specimens as described elsewhere (Medici MC et al., 2005) and used for the probe evaluation.

NoV PCR was carried out targeting the (ORF1)-ORF2 junction region, (T7_GII_FOR: 5'-aattctaatacgactcactatagggagaaggCAAGAGCCAATGTTCAGATGGATG-3', GII_REV: 5'-CGTCATTCGACGCCATCTTCA-3') whereas the amplification of the rotavirus samples was performed targeting a fragment of the gene coding for the VP7 protein (SBeg9: 5'-GGCTTTAAAAGAGAGAATTTC-3', T7_9T1-1: 5'-aattctaatacgactcactatagggagaagg TCTTGTCAAAGCAAATAATG-3'). The amplified tract of NoV and RV genomes results in a 135 bp and a 235 bp amplicons respectively. A T7 polymerase sequence (Rutjes SA et al., 2006) was appended at a 5' end of specific primers (upper-case letters: primer sequence; lower-case letters: additional T7 promoter sequence) in order to carry out the isothermal amplification. PCR amplifications were performed in a final volume of 50 μl, using a PCR System 9700 thermal cycler (Perkin Elmer Applied Biosystems) starting from 10 μl of extracted RNA. The single-tube RT-PCR was performed using the "SuperScriptTM One-Step RT-PCR for Long Templates" (Invitrogen, California, USA), and 0.2 μM primers. The PCR program was as follow: i) RT step: 58 °C for 30 min ii) amplification step: initial denaturation at 94 °C for 2 min followed by 40 cycles as follow: 94 °C for 30 sec, 58 °C for 1 min and 68°C for 1 min; and a final extension step at 68 °C for 5 min.

The isothermal ampification step was carried out in a final volume of 20 μl using the Riboprobe® in vitro Transcription Systems kit (Promega) according to the manufacturer's instruction. The master mix contained 2.5 mM rNTPs, 100mM DTT, 20 U T7 RNA polymerase, 1X transcription optimized buffer and 100 nM or 200 nM PNA probes,

depending on the esperiment . For positive samples, PCR amplicons were added (18ng of NoV, 8ng of Rotavirus) in order to give a comparable amount of transcripts at the end of the reaction. All the reactions were performed in 384 well fluorescence plates and at 40 °C for 35 min. The fluorescence emission was monitored with a Victor 3 plate reader selecting the fluorescein excitation and emission filters (Excitation Filter*:* 485 nm. Emission Filter: 530 nm).

3.3. RESULTS AND DISCUSSION

3.3.1 Probe design

The high genetic diversity of noroviruses due to the high mutation rate makes the design of specific and effective primers and probes a challenging task. The most common strategies employed to overcome this major obstacle rely on the incorporation of degenerate bases or involve the contemporary use of multiple primer and probe sets (Kageyama T et al., 2003; Höhne M and Schreier E, 2004; Jothikumar N et al., 2005; Mohamed N et al., 2006).

However, other proposed approaches for the optimization of sequences design for NoV detection are based on chemically modified probes such as minor groove binder (MGB) probes (Höhne M and Schreier E, 2004; Hymas W et al., 2007) or locked nucleic acid (LNA) probes (Dreier J et al., 2006); this chemical modification allow the design of shorter, highly specific probes without losses in thermal stability. Furthermore, the reduction of the length of the targeted sequences increases the chance to identify short and highly conserved nucleotide stretches, which might result inadequate to be targeted with conventional probes. Surprisingly, all the previously reported strategies for the modified probe design were optimized for PCR-based assays and no examples are available for isothermal-based amplification methods, which showed to be reliable alternatives to PCR in NoV detection (Moore C et al., 2004; Houde A et al., 2006; Patterson SS et al., 2006; Rutjes SA et al., 2006; Fukuda S et al., 2008; Iturriza-Gómara M et al., 2008; Yoda T et al., 2009).

The employment of PNA-based probes for NoV detection relies basically on these considerations with the major aim to develop a PNA probe suitable for specific detection in isothermal conditions. In order to identify a short, highly conserved stretch of nucleotide along the highly conserved ORF1-ORF2 junction region (Kageyama T et al., 2003), all the available NoV GGII sequences were retrived, alligned and investigated for the presence of highly conserved stretches of 10-15 nucleobases: this range was defined according to the

usually employed optimal PNA length in order to ensure the required specificity and thermal stability. The different sequence variants identified within the selected portion of the NoV genome and their respective frequencies were taken into account in order to design a broadly reactive PNA probe (Table 3.1). According to the data resulting from the alignment of approximately 800 sequences, the sequence defined for the PNA probe targets with full-match precision approximately the 98% of the all represented sequences. Among the different strategies suitable for the integration of the PNA probes into real time isothermal-based assays, the conjugation with thiazole orange appeared to be the best solutions for several reasons: i) the method relies on the fluorescence enhancement upon hybridization of the single asymmetric cyanine dye and does not require any coupling with quenching molecules; this single-fluorophore based approach result in an easier design compared to other strategies, such as the PNA molecular beacons; ii) the possibility to tether the thiazole orange at the end of the PNA probe or directly bind it to the PNA backbone replacing a nucleobase provide a flexible strategy that can be easily adapted to the different purposes of various assays. The FIT approach, for example, might be an effective solution to obtain mismatch-tolerant probes: since the thiazole orange linked to the PNA backbone behaves like a universal base, the substitution of the probe nucleobase facing the most relevant source of point-mutations might broaden the detection rate without affecting the fluorescence emission; iii) the emission wavelength of the TO molecule is compatible with the majority of the commercially available real time PCR instruments, fluorimeters and plate readers.

Although Light-Up and FIT strategies were accurately studied by their respective inventors, few real applications using these kind of probes have been currently developed; moreover, comparisons of the performances of the two technologies with synthetic oligonucleotides or real samples are not yet reported. In order to assess the applicability of both the TO-based strategies for NoV detection, two PNA probes with the same sequence but differing for the TO conjugation approach (Table 3.2), were synthesized and evaluated in an isothermal assay.

The use of thiazole orange as fluorophore is compatible with the solid phase synthesis protocols commonly applied in the synthesis of PNAs, since this molecule is stable under the strong acidic conditions used, in particular in the final step which allows to obtain the oligomer cleaved from the solid support. Both the PNA sequences were synthesized by an automatic peptide synthesizer following Boc synthesis protocols.

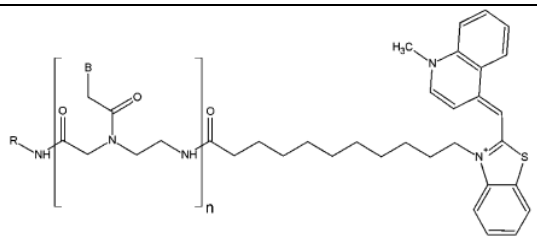
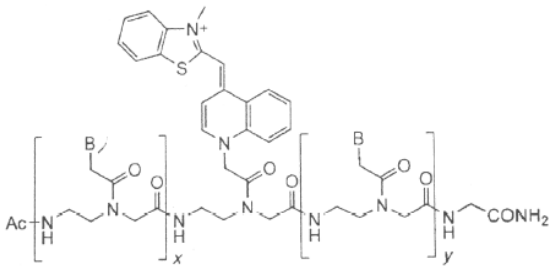
Table 3.1 Probe design: viariants of norovirus GII retrieved and alligned from GeneBank, with their respective frequencies. (X87557: sequence used for the alignement); the sequence identified for probes design is marked in yellow

Sequences	**Frequency**
agattctcagatctgagcacgtgggagggcgatcgcaatctggctcccagctttgtgaa	1
..........t.....	298
..g..t..........t.....	56
..........t.....t.....	55
..g.........a....a.....t.....	40
.......g.....t.....	31
..g..t..t..ct.....t.....	24
.......t..c..c.....t.....t......gaagg.....	21
..g.....ct.....	18
..........c.....t.....	18
..........t.....t.....tc.....	18
.......t..c..c.....a.....t......gaggg.....	16
.......t..ct.....t.....	15
..........ct.....t.....	14
..........c..c..t..t.....t......gaagg.....	14
.......t..c..c.....a.....t......gagag.....	13
..g.....t.....	10
.......t..c..c.....a.....t......gaagg.....	10
.....	10
....t.....c..c.....gagaa.....	7
..........t.....t.c.....	6
....t.....c..c.....tgg.....	5
..g..t.....c.....t.....	4
..........t.....t.....t.....tc.....	4
..g..t..t..c.....t.....	3
.......t..c..c..t..t.....t......gaagg.....	3
..g..t..t..c..c.....gaggg.....	2
..g.....t..ct.....t.....	2
..g.....t.....t.....	2
..g.....a.....t.....	2
....t.....c..c.....a.....t......gatgg.....	2
.......t..c..c.....a.....t.....t......gaggg.....	2
.......t..c..c.....a.....t.....t......gatgg.....	2
..........c..c.....atgg.....	2
..........c..c.....tgg.....	2
.....a.....t.....	2
.....t..c.....	2
..g..tc.....t...t.....	1
..g..t..t..ct.....t.....t.....	1
..g..t..t..ct.....a.....t.....	1
..g..t..t..ct.....	1
..g..t....g.....t.....	1
..g..t.....t.....t.....	1
..g.....g.....t.....	1
..g.....t..ct.....g.t.....	1
..g.....t..ct.....	1
..g.....t..c..c.....t.....t......gaagg.....	1
..g.....ct.....t.....	1
..g.....c.....t.....	1
..g.....a...c.a.....t.....	1
..g.....a.....a.....ct....t.....	1
..g.....a.....a.....c.....t.....	1
..t.....t.....	1
...g.....t.....	1
....t..t..c..c.....a.....t......gaagg.....	1
....t.....c..c.....a.....gatgg.....	1
....t.....c..c.....a.....tgg.....	1
....t.....c..c.....a.....tgg.....	1
....t.....t.....t.....tc.....	1
....t.....t.....t.....	1
....t.....t.....	1
.....c.t..ct.....t.....	1
.....c.....t.....	1
.....g.....t.....	1
.....g.....t.....t.....	1
.....g.....t.....tc.....	1
.....t..c..c.....a.....g.....gag.a.....	1
.....t..c..c.....a.....t......gaaag.....	1
.....t..c..c.....t.....t.....t......gaagg.....	1
.....t..c..c.....t.....gaggg.....	1
.....t..c..c.....t......gaagg.....	1
.....t..c..c.....ga.gg.....	1
.....g.....t.....	1
..........ct.....t.....g.....g....c.....gat.....	1
.....c.c.....t.....	1
.....c..c..t..ta.....t......gaagg.....	1
.....c.....t.....g.....g....c.....gat.....	1
.....c.....a.....t.....	1
.....t.....t.....tc.....	1
.....t.....t.....g.....g....c.....gat.....	1
.....t.....a.....t.....	1
.....t.....t.....t.....	1
.....t.....t.....t.cc.....	1
.....t.....	1
.....c.....t.....	1
.....g.....t.....	1
.....c.....t.....	1
.....t.....t.....	1
.....a.....t.....	1
.....c.....t.....	1
.....a.....t.....	1
.....a.....t.....	1
.....g.....t.....	1
.....t.....tc.....	1
.....t.....	1
.....t..t.....	1

GGGAGGGCGA	777	(97.7%)
.......**T**..	9	(1.1%)
....**A**.....	4	(0.5%)
.......**G**..	3	(0.4%)
....**C**.....	1	(0.1%)
.......**A**..	1	(0.1%)

LightUp-PNA was obtained by conjugating the N-terminal of PNA sequence with 6-TO exanoic acid. This reaction was performed by using the DIC/DhBTOH coupling protocol and with an extensive coupling time, since the reaction is slow for the high steric hyndrance.
Concerning the FIT-PNA probe, the thiazole orange derivative used is 2-TO ethanoic acid and it was covalently linked to the PNA backbone in the chosen position in the same experimental conditions performed for the Light-Up probe. Both the thiazole derivatives, were previously synthesized in the organic chemistry laboratories of the University of Parma.
Before testing their performances, the two PNA probes were purified by semipreparative RP-HPLC and characterized by ESI mass spectrometry.

Table 3.2. TO-modified PNA probes used in this study

Probe sequence (H-NH_2)	TO- conjugation strategy
LightUp_NoVGII: (TO)-TCGCCCTCCC	
FIT_NoVGII: TCGCC-(TO)-TCCC	

3.3.2 Thermal stability

The thermal stability of the TO-conjugated probes with nucleic acids was evaluated by melting curve analyses. The melting temperature (T_m) values were calculated for each probe using complementary DNA and RNA synthetic oligonucleotides and the effect of the linked TO- molecule was estimated by comparison with the PNA unmodified probe (Table 3.3). In accordance with the previously reported results, both the PNA probes showed higher T_m values upon hybridization with the RNA than with DNA: the unmodified probe showed the more pronounced ΔT_m (13°C) followed by the LighUp_NoVGII (11°C) and the FIT_NoVGII (9°C).

Interestingly, the TO molecule employed as end-labelling fluorophore seems to play a strong stabilizing effect upon hybridization with both the DNA and the RNA targets (+10 °C and +8 °C), compared to the unmodified PNA probe (PNA_10mer). When the TO moiety is directly linked to the PNA backbone replacing a nucleobase, the stabilizing effect was found to be less pronounced with DNA (3 °C) and completely disappeared upon hybridization with RNA, which appeared even slightly destabilized (-1°C).

Table 3.3. Melting temperatures of PNAs with oligonucleotides

Probe	oligonucleotides		
	DNA (°C)	**RNA (°C)**	**ΔT_m (°C)** [b]
LightUp_NoVGII	67 (+10) [a]	78 (+8) [a]	11
FIT_NoVGII	60 (+3) [a]	69 (-1) [a]	9
PNA_10mer	57	70	13

Notes: (a) The ΔT_m values between the TO-conjugated probes and the unmodified probe are reported in brackets. (b) ΔT_m values calculated for each probe, as difference of the PNA T_m values of hybridized with the complementary RNA and DNA oligonucleotides.

Furthermore, the higher T_m values observed for the LighUp_NoVGII probe might be due to the presence of an additional nucleobase compared to the FIT_NoVGII probe, since the former consists of ten nucleobases and a single TO molecule, whereas the latter is composed by nine nucleobases and a single TO molecule. However, the most relevant observation is the strong stabilizing effect played by the TO moiety in the LighUp_NoVGII probe as compared with the unmodified probe: this effect (+10°C and +8 °C upon hybridization with DNA and RNA) seems to be comparable and even higher than the thermal stability contribution given by a conventional nucleobase.

3.3.3 Fluorescence measurements

Despite TO-conjugated PNA probes were successfully employed for specific nucleic acid recognition, a major drawback of this approach is still the lack of general rules for the estimation of the probe fluorescence behaviour, thus fullfilling the needs for a robust probe design. According to the available data, the fluorescence emission of the free probes and the hybridized probes, changes depending on the probe sequence and the nucleobase environment that surround the TO position (Svanvik N et al., 2000a; Köhler O et al., 2005). However, no

predictive analyses can be effectively performed during the design phase. In order to test the performances of the probes synthesized, their fluorescence properties were initially evaluated using synthetic DNA oligonucleotides (Figure 3.3).

The probes were evaluated with complementary and noncomplementary oligonucleotides at different temperatures (25 °C and 45 °C).

The oligonucleotides used for the T_m studies were perfectly complementary and with the same length of the probes studied and did not bear any overhang sequences. Surprisingly, the LighUp_NoVGII did not showed increased fluorescence emissions upon hybridization using oligonucleotides with no overhang sequences (data not shown), whereas the T_m studies showed similar stabilizing contributions with longer oligonucleotides.

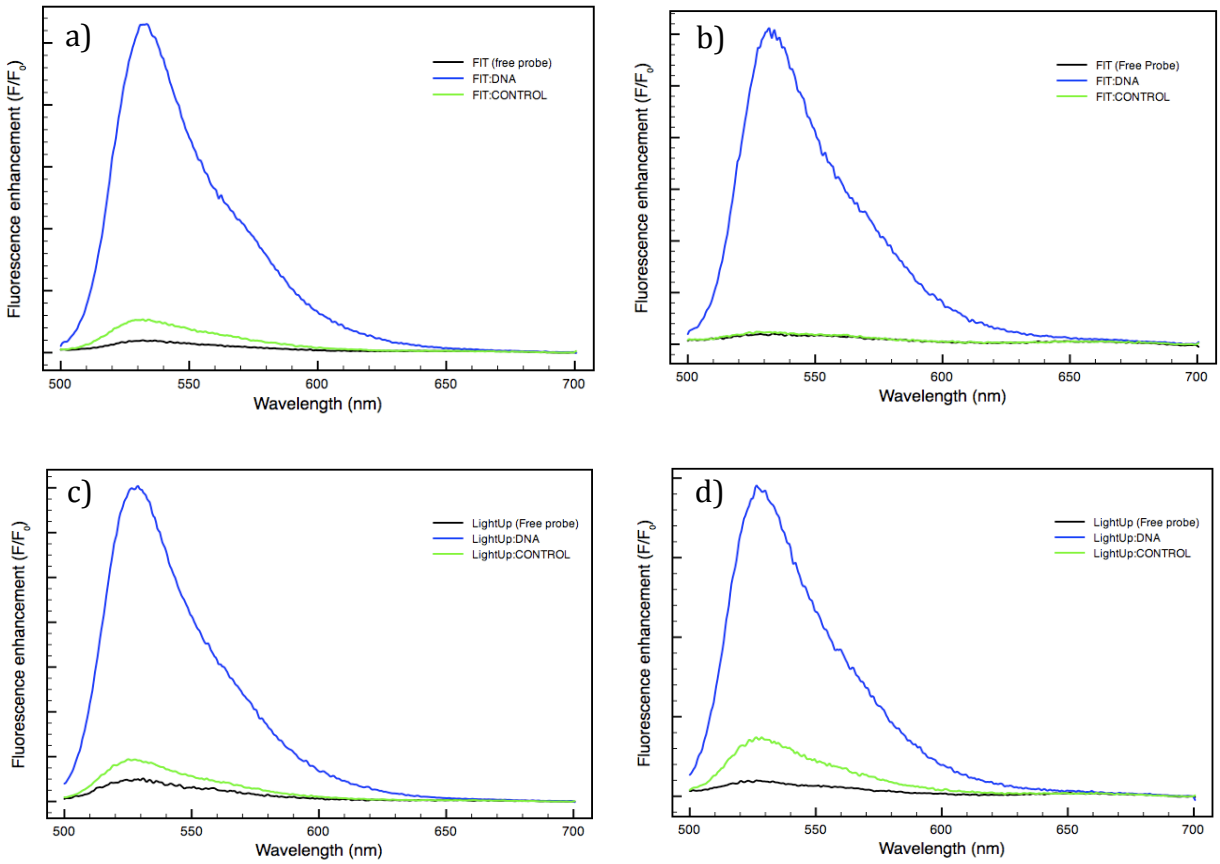


Figure 3.3. a) Fluorescence emission spectra of free FIT_NoVGII (black line) and FIT_NoVGII in the presence of complementary (blue line) and noncomplementary (green line) DNA oligonucleotides at 25 °C and b) 45 °C. c) fluorescence emission spectra of free LightUp_NoVGII (black line) and LightUp_NoVGII in the presence of complementary (blue line) and noncomplementary (green line) DNA oligonucleotides at 25 °C and d) 45 °C. The DNA sequences were 5'-ATCTGAGCACGTGGGAGGGCGATCGCAATCTGGCTC-3' (complementary) and CACGGTTTGGAACACCACAAT (noncomplementary)

These diffent behaviours suggest the existence of various mechanisms of interaction between the TO and the nucleic acids upon probe hybridization. Since no strong variations in the fluorescence emission were observed for the LightUp_NoVGII probe using a complementary decamer oligonucleotide, a longer oligonucleotide with overhangs at both sides was used for the measurements. A similar strategy was employed in previous works reported (Svanvik et al., 2000a).

The same oligonucleotide was used with the FIT_NoVGII probe; however, in order to estimate potential effects of the additional nucleobases on the fluorescence enhancement, the FIT_NoVGII probe was also studied with a decamer oligonucleotide; no significant differences in fluorescence emission upon hybridization were observed (data not shown). The results are summarized in Table 3.4. Both probes, hybridized with the complementary oligonucleotide, showed increased fluorescence emission and modest aspecific interactions were observed in the presence of the noncomplementary oligonucleotide. However, FIT_NoVGII exhibits a higher fluorescence enhancement upon specific hybridization (28.0- and 28.8-fold vs 14.0- and 19.5-fold of the LightUp_NoVGII at 25 °C and 45 °C respectively). Interestingly, at higher temperature, the aspecific signal tends to decrease for FIT_NoVGII but seems to increase for LightUp_NoVGII.

This effect is mainly due to a lower decrease in fluorescence emission for the "PNA probe:noncomplementary DNA oligonucleotide complex" compared to the signals of the free probe, when the temperature is raised from 25 °C to 45 °C. According to these results, the target discrimination efficiency seems to be better for FIT_NoVGII, especially at 45 °C.

Table 3.4. Fluorescence enhancement upon hybridization for FIT_NoVGII and LightUp_NoVGII using complementary and noncomplementary oligonucleotides

	FIT_NoVGII		LighUp_NoVGII	
	(25° C)	(45° C)	(25° C)	(45° C)
compl	28.0	28.8	14.0	19.5
noncompl	2.6	1.2	1.8	3.7

The ability to provide good emission signals at high temperature is a key feature of fluorescent systems employed in real time detection assays, especially for PCR-based

methods, wherein the probe hybridization usually occurs during the annealing step, usually performed at elevated temperatures, at 50-70 °C.

In order to evaluate the effect of the temperature on the fluorescence emission, the two PNA probes hybridized with the complementary oligonucleotide, were studied between 25 °C and 58 °C (Figure 3.4). Both probes showed a strong decrease in fluorescence intensities when the temperature is increased: at 58 °C the fluorescence emission appeared reduced to the 13% and 23% of the initial values for FIT_NoVGII and LightUp_NoVGII respectively. The intensities decreased linearly with the exception of the LightUp_NoVGII probe, which seems to decrease slower in the higher range of temperatures tested, between 48 °C and 58°C. This rapid temperature-dependent decrease in the fluorescence emission might be primarly due to the increased thermal motion of the DNA bases, which increases the possibility of the TO molecule to move in its interaction site, thus increasing the efficiency of the non radiative events (Nygren J et al., 1998). Similar decreasing rates for end-labelled PNA-TO conjugates were observed in previous works (Svanvik et al., 2000a).

These results suggest that the real time PCR-based applications might not be the most suitable type of assays for the TO-conjugated PNA probes because of the high temperatures (55-70 °C) required during the signal acquisition steps.

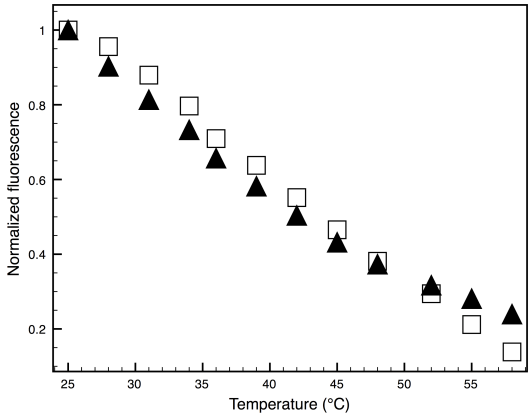


Figure 3.4. Fluorescence emission of FIT_NoVGII (□) and LightUp_NoVGII (▲) in presence of complementary oligonucleotide as function of temperature

The integration of this kind of probes with the isothermal-based methods, which usually work in a lower temperature range (37-41 °C) might be an effective strategy to maximize the emission efficiency upon hybridization of the TO-PNA probes. Moreover, the lower operative temperatures employed in the isothermal assays make more difficult the design of highly selective and specific probes and require accurate evaluations on target accessibility, due to

the presence of stable secondary structures which result particularly relevant for RNA targeting (Nadal A et al., 2007). The use of PNA probes might be an effective strategy to minimize or overcome these limitations (Armitage BA, 2003).

Performances in detecting RNA, were studied using the FIT_NoVGII probe in the presence of the complementary RNA oligonucleotide (Figure 3.5). The absolute values of the fluorescence emission upon RNA hybridization resulted higher than the signals obtained for the analogous DNA oligonucleotide, as well as the fluorescence enhancement (42- and 60-fold at 25 °C and 45 °C respectively). The higher fluorescence intensities targeting the RNA, along with the better performances at higher temperature (45 °C), support the idea that TO-PNA probes are particularly suitable for isothermal-based amplification assays performed at a constant temperature of 37-41 °C and targeting RNA amplicons.

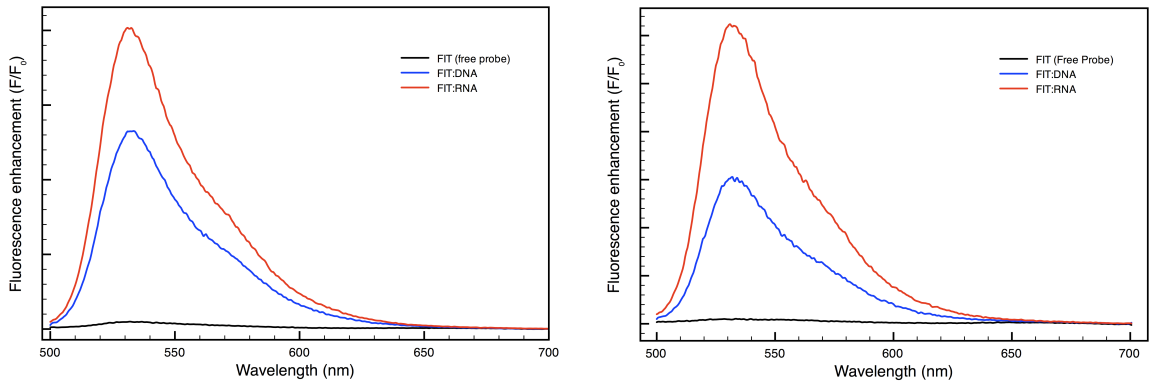


Figure 3.5. Fluorescence emission spectra of free FIT_NoVGII (black line) and FIT_NoVGII in the presence of complementary DNA (blue line) and complementary RNA (red line) oligonucleotides at 25 °C (*left*) and 45 °C (*right*). The DNA and RNA sequences were 5'-GGGAGGGCGA -3'

3.3.4 Target RNA detection using a Real Time Isothermal Amplification System

In order to evaluate the TO-PNA probes as tools for NoV RNA detection in clinical specimens using isothermal amplification assays, a two-step amplification-transcription system, based on a PCR amplification step followed by a subsequent T7 polymerase transcription of the amplified target, was developed and used as model to evaluate TO-PNA probes/amplified RNA interaction (Figure 3.6). In this method, a PCR primer bearing the specific T7 promoter sequence was used to introduce the additional sequence into the amplicon. The first-round PCR amplicons were then transcribed *in vitro* as RNA molecules during a continuous reaction at constant temperature (40 °C) in the presence of a T7 polymerase. The transcription phase was optimized in order to provide amplification rates

comparable to the NASBA assays (Perlin DS and Zhao Y, 2008; Lau LT et al., 2006) resulting in a final amount of 10^{12} - 10^{14} target molecules.

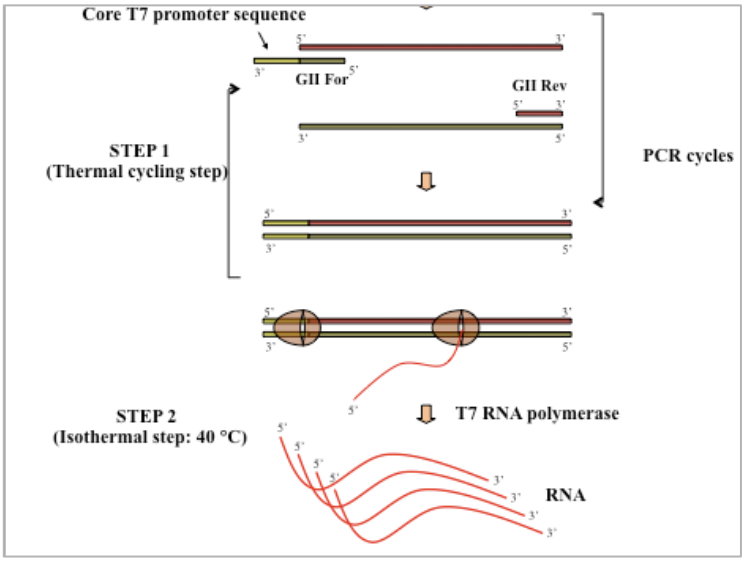


Figure 3.6. Scheme for the amplification strategy employed to detect NoV samples extracted from stool specimen. The T7 promoter sequence was introduced with PCR amplification (STEP 1) and an aliquot of the amplified product was used for the T7 transcription reaction (STEP 2). The RNA products were detected in 20 ul using theTO-conjugates PNA probes

All the samples used in this study were collected and previously tested as positive for NoV or RV using internally developed PCR methods by the Department of Pathology and Laboratory Medicine, Section of Microbiology of the University of Parma (Medici MC et al., 2003).

Two different probes concentrations (0.1 μM and 0.2 μM) were evaluated (Figure 3.7). In order to properly compare the signals obtained from the transcription reactions, all the samples were quantified using an RNA-specific quantification assay; the TO-PNA probes do not interfere with the fluorophore provided with the ready-to-use kit, which has the excitation and emission maxima more than 100 nm above the TO (λ_{ex}=644; λ_{ex}=673, according to the manufacturer's technical specifications). However, all the samples were also tested at *time zero* (before the addition of the T7 polymerase) to verify potential probe interferences; all the values measured resulted under the detection limit of the employed kit. In order to test the aspecific signals of the TO-PNA probes in the presence of large amounts of non-target RNA, samples of rotavirus (RV) were treated in the same way: samples were amplified using specific primers, one of which containing the T7 promoter sequence. An amplicons aliquot was then used in the isothermal transcription system in the presence of the TO-PNA probe, in order to obtain an amount of RNA comparable to that detected for NoV samples. The assay

was optimized in order to give comparable amounts of transcribed RNA for both NoV and RV samples, using a known amount of the initial dsDNA as template for the isothermal step, attempting to minimize as much as possible the variability of the fluorescence signals due to dramatically different transcription rates. The samples used for signal comparison showed a comparable amount of RNA at the end of the transcription reaction, ranging from 20 to 30 ng/µl.

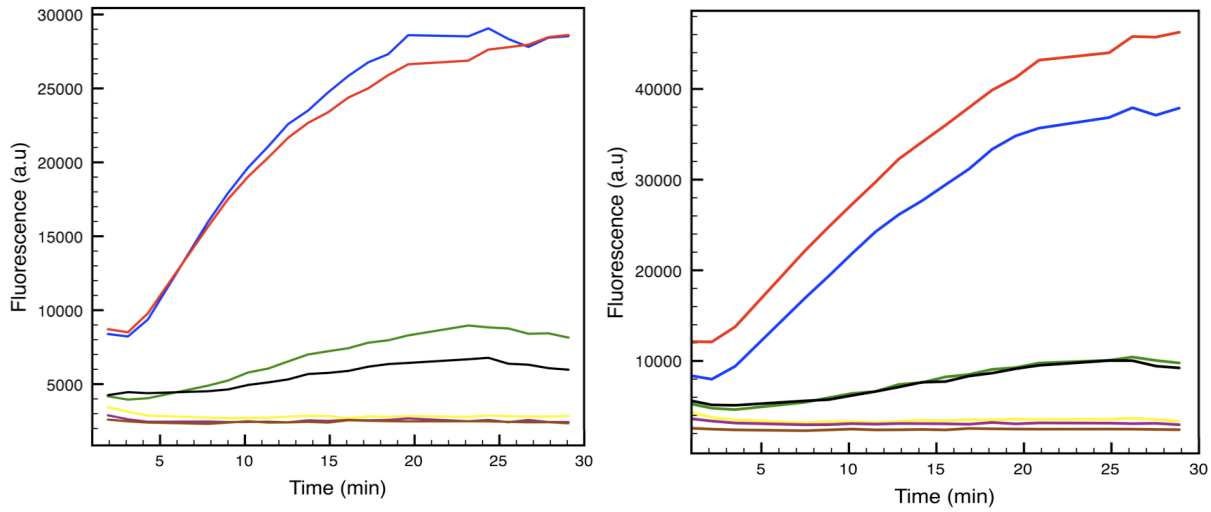


Figure 3.7. Real time detection of RNA transcription using probe concentrations of 0.1 µM (*left*) or 0.2 µM (*right*). The fluoresence signals of FIT_NoV_GII alone (yellow) and in the presence of norovirus (blue) or rotavirus (green) and LightUp_NoV_GII alone (violet) and in the presence of norovirus (red) or rotavirus (black), along with the background signal of the buffer alone (brown) are reported.

According to the results obtained, both FIT_NoVGII and LightUp_NoVGII allowed to monitor the trascription events in real time and both probes exhibited a good fluoresence enhancement in the presence of the target RNA. Moreover, the fluorescence emissions in presence of high concentrations of nontarget RNA never increased more than 3-fold, showing a good specificity. The probe concentration of 0.1 µM resulted sufficient to provide a detectable fluorescent signal and increasing the probe concentration up to 0.2 µM did not significantly improve the $F_{specific}/F_{aspecific}$ ratio. It has to be noticed that, despite the good selectivity showed by the TO-PNA probes, the fluorescent enhancement targeting the transcribed NoV RNA resulted lower when compared to the data observed using the synthetic oligonucleotides. This might be due to differences in the sequence accessibility targeting a longer RNA at a constant temperature of 39-40 °C. However, the aspecific florescence detected in this experiment represents the *worst-case scenario* in which the transcription of

aspecific RNA (rotavirus) is efficient as the specific target and provide a comparable amounts of RNA at the end of the assay.

These results suggest that the TO-conjugated PNA probes can be effective tools for the development of isothermal assays for NoV analysis. Moreover, this might also open intersting possibilities for the integration of PNAs in special applications such as analysis of RNA genomes or specific sequences (mRNA, miRNA) or SNPs detection on RNA strands. The employment of one-step, single-tube isothermal systems will be surely taken into account for future studies. The two-step amplification-transcription system used in this work was designed to be easily controlled and used without the employment of elaborate enzymes mixture and it does not represent the best solution in terms of performances, costs and time-to-results. However, a similar solution was employed to improve detection sensitivity as alternative to nested PCR (Jean J et al., 2003). This application is to our knowledge the first example of TO-conjugated probes for specific RNA detection using an isothermal assay.

3.4 CONCLUSIONS

Two different TO-conjugated PNA probes were developed and successfully employed for NoV GII detection in an isothermal-based assay. The TO-PNA probes showed a good fluorescence enhancement upon hybridization with the target RNA sequence allowing the real time monitoring of the transcription-based amplification. Moreover, even in the presence of nontarget RNA (rotavirus) in amounts comparable to the RNA obtained for the target sequence, the probe fluorescence due to aspecific interactions remains low, never exceding the 3-fold increase, compared to the fluorescence of the free probes. According to the results obtained, the PNA probes conjugated with dyes like the TO can be used in isothermal assays for specific RNA targeting of NoV samples. Although the FIT_NoVGII showed slightly better fluorescence performaces upon hybridization with oligonucleotides than LightUp_NoVGII, both probes showed similar behaviours during the real time detection of the transcribed RNA obtained in the isothermal assay. These results suggest that both the FIT and the Ligh-Up approaches are suitable for targeting RNA in isothermal-based amplification assays.

3.5 REFERENCES

Armitage BA (2003). The impact of nucleic acid secondary structure on PNA hybridization. Drug Discov Today;8(5):222-8

Bethge L, Jarikote DV, Seitz O (2008). New cyanine dyes as base surrogates in PNA: forced intercalation probes (FIT-probes) for homogeneous SNP detection. Bioorg Med Chem;16(1):114-25.

Deiman B, van Aarle P, Sillekens P (2002). Characteristics and applications of nucleic acid sequence-based amplification (NASBA). Mol Biotechnol;20(2):163-79.

Dreier J, Störmer M, Mäde D, Burkhardt S, Kleesiek K (2006). Enhanced reverse transcription-PCR assay for detection of norovirus genogroup I. J Clin Microbiol;44(8):2714-20.

Fukuda S, Sasaki Y, Seno M (2008). Rapid and sensitive detection of norovirus genomes in oysters by a two-step isothermal amplification assay system combining nucleic acid sequence-based amplification and reverse transcription-loop-mediated isothermal amplification assays. Appl Environ Microbiol;74(12):3912-4

Houde A, Leblanc D, Poitras E, Ward P, Brassard J, Simard C, Trottier YL (2006). Comparative evaluation of RT-PCR, nucleic acid sequence-based amplification (NASBA) and real-time RT-PCR for detection of noroviruses in faecal material. J Virol Methods;135(2):163-72

Hymas W, Atkinson A, Stevenson J, Hillyard D (2007). Use of modified oligonucleotides to compensate for sequence polymorphisms in the real-time detection of norovirus. J Virol Methods.;142(1-2):10-4.

Höhne M, Schreier E (2004). Detection and characterization of norovirus outbreaks in Germany: application of a one-tube RT-PCR using a fluorogenic real-time detection system. J Med Virol; 72(2):312-9

Isacsson J, Cao H, Ohlsson L, Nordgren S, Svanvik N, Westman G, Kubista M, Sjöback R, Sehlstedt U (2000). Rapid and specific detection of PCR products using light-up probes. Mol Cell Probes;14(5):321-8.

Iturriza-Gómara M, Xerry J, Gallimore CI, Dockery C, Gray J (2008). Evaluation of the Loopamp (loop-mediated isothermal amplification) kit for detecting Norovirus RNA in faecal samples. J Clin Virol;42(4):389-93.

Jean J, D'Souza D, Jaykus LA (2003). Transcriptional enhancement of RT-PCR for rapid and sensitive detection of Noroviruses. FEMS Microbiol Lett;226(2):339-45.

Jothikumar N, Lowther JA, Henshilwood K, Lees DN, Hill VR, Vinjé J (2005). Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. Appl Environ Microbiol;71(4):1870-5.

Keer JT, Birch L (2003). Molecular methods for the assessment of bacterial viability. J Microbiol Methods;53(2):175-83.

Kou X, Wu Q, Zhang J, Fan H (2006). Rapid detection of noroviruses in fecal samples and shellfish by nucleic acid sequence-based amplification. J Microbiol;44(4):403-8.

Köhler O, Jarikote DV, Seitz O (2005). Forced intercalation probes (FIT Probes): thiazole orange as a fluorescent base in peptide nucleic acids for homogeneous single-nucleotide-polymorphism detection. Chembiochem;6(1):69-77.

Köhler O, Seitz O (2003). Thiazole orange as fluorescent universal base in peptide nucleic acids. Chem Commun;(23):2938-9.

Lau LT, Fung YW, Yu AC (2006). Detection of animal viruses using nucleic acid sequence-based amplification (NASBA). Dev Biol (Basel).;126:7-15.

Leijon M, Mousavi-Jazi M, Kubista M (2006). LightUp probes in clinical diagnostics. Mol Aspects Med. Apr-Jun;27(2-3):160-75.

LightUp website: www.lightup.se.

Mackay J (2007), Landt O. Real-time PCR fluorescent chemistries. Methods Mol Biol;353:237-61

Medici MC, Martinelli M, Ruggeri FM, Abelli LA, Bosco S, Arcangeletti MC, Pinardi F, De Conto F, Calderaro A, Chezzi C, Dettori G (2005). Broadly reactive nested reverse transcription-PCR using an internal RNA standard control for detection of noroviruses in stool samples. J Clin Microbiol;43(8):3772-8.

Mohamed N, Belák S, Hedlund KO, Blomberg J (2006). Experience from the development of a diagnostic single tube real-time PCR for human caliciviruses, Norovirus genogroups I and II. J Virol Methods;132(1-2):69-76.

Moore C, Clark EM, Gallimore CI, Corden SA, Gray JJ, Westmoreland D (2004). Evaluation of a broadly reactive nucleic acid sequence based amplification assay for the detection of noroviruses in faecal material. J Clin Virol;29(4):290-6.

Mori Y, Notomi T (2009). Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. J Infect Chemother;15(2):62-9.

Nadal A, Coll A, Cook N, Pla M (2007). A molecular beacon-based real time NASBA assay for detection of Listeria monocytogenes in food products: role of target mRNA secondary structure on NASBA design. J Microbiol Methods;68(3):623-32.

Nygren J, Svanvik N, Kubista M (1998). The interactions between the fluorescent dye thiazole orange and DNA. Biopolymers;46(1):39-51.

Parida M, Sannarangaiah S, Dash PK, Rao PV, Morita K (2008). Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. Rev Med Virol;18(6):407-21.

Patel MM, Hall AJ, Vinjé J, Parashar UD (2009). Noroviruses: a comprehensive review. J Clin Virol;44(1):1-8.

Patterson SS, Smith MW, Casper ET, Huffman D, Stark L, Fries D, Paul JH (2006). A nucleic acid sequence-based amplification assay for real-time detection of norovirus genogroup II. J Appl Microbiol;101(4):956-63

Perlin DS, Zhao Y (2009). Molecular diagnostic platforms for detecting Aspergillus. Med Mycol.;47 Suppl 1:S223-32.

Ranasinghe RT, Brown T (2005). Fluorescence based strategies for genetic analysis. Chem Commun;(44):5487-502

Rutjes SA, van den Berg HH, Lodder WJ, de Roda Husman AM (2006). Real-time detection of noroviruses in surface water by use of a broadly reactive nucleic acid sequence-based amplification assay. Appl Environ Microbiol;72(8):5349-58.

Saito Y, Kanatani K, Ochi Y, Okamoto A, Saito I (2004). Design of base-discriminating fluorescent (BDF) nucleobase for SNP typing. Nucleic Acids Symp Ser;(48):243-4

Socher E, Jarikote DV, Knoll A, Röglin L, Burmeister J, Seitz O (2008). FIT probes: peptide nucleic acid probes with a fluorescent base surrogate enable real-time DNA quantification and single nucleotide polymorphism discovery. Anal Biochem;375(2):318-30.

Svanvik N, Ståhlberg A, Sehlstedt U, Sjöback R, Kubista M (2000). Detection of PCR products in real time using light-up probes. Anal Biochem;287(1):179-82.

Svanvik N, Westman G, Wang D, Kubista M (2000). Light-up probes: thiazole orange-conjugated peptide nucleic acid for detection of target nucleic acid in homogeneous solution. Anal Biochem;281(1):26-35.

Venkatesan N, Seo YJ, Kim BH (2008). Quencher-free molecular beacons: a new strategy in fluorescence based nucleic acid analysis. Chem Soc Rev;37(4):648-63

Weile J, Knabbe C (2009). Current applications and future trends of molecular diagnostics in clinical bacteriology. Anal Bioanal Chem;394(3):731-42.

Wirgart BZ, Andersson P, Grillner L (2005). Evaluation of the ReSSQ assay in relation to the COBAS AMPLICOR CMV MONITOR test and an in-house nested PCR method for detection of cytomegalovirus DNA. J Clin Microbiol;43(8):4057-63.

Wolffs P, Knutsson R, Sjöback R, Rådström P (2001). PNA-based light-up probes for real-time detection of sequence-specific PCR products. Biotechniques;31(4):766, 769-71.

Yoda T, Suzuki Y, Yamazaki K, Sakon N, Kanki M, Kase T, Takahashi K, Inoue K (2009). Application of a modified loop-mediated isothermal amplification kit for detecting Norovirus genogroups I and II. J Med Virol;81(12):2072-8.

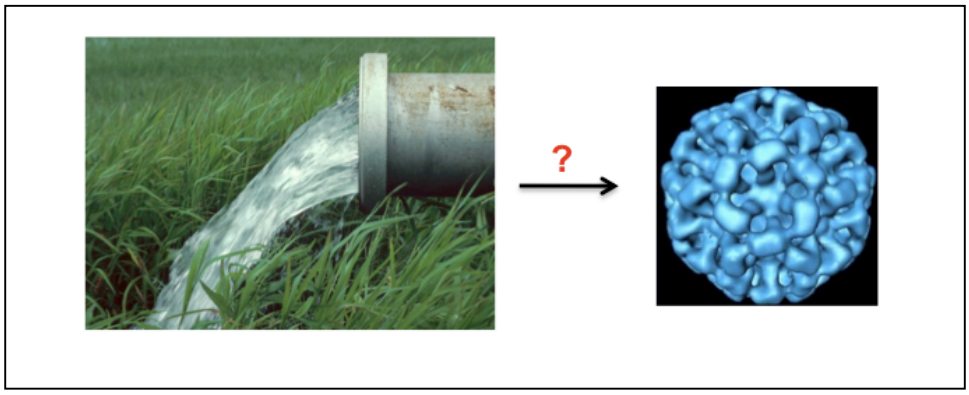
Zhao W, Ali MM, Brook MA, Li Y (2008). Rolling circle amplification: applications in nanotechnology and biodetection with functional nucleic acids. Angew Chem Int Ed;47(34):6330-7.

Chapter 4

Detection of Norovirus in water: feasibility study for a new filtration device

Among the causes of norovirus spread, water seems to play a relevant role. Since effective methods for water-borne viruse recovery and detection are still on development and the current methods reported are often expensive, time-consuming and requiring cumbersome operations to be performed, the monitoring of viruses in water on routine bases appears to be beyond the capability of many private and public organizations responsible for drinking-water safety.

In this context, a new glass wool-based filtration device was investigated as tool for rapid norovirus analysis in water.



Declaration: this project was developed at the 'Centre Christophe Mérieux' for molecular biology and microsystems (bioMérieux SA, Grenoble, France). Since some informations must not be disclosed, this chapter is based on a more detailed document covered by a confidential agreement and any lack of details in the experimental procedures has to be considered in this context.

4.1 INTRODUCTION

Among the causes of NoV spread, water seems to play a relevant role. The Center for Desease Control and Prevention reported the role of water in the 3% of the total cases of gastroenteritis (348) caused by NoVs between January 1996 and November 2000; other data on water-borne disease outbreaks acquired from January 2001 to December 2002 reported NoVs as the cause of outbreaks in 26% of the total cases associated with pathogens, excluding acute chemical poisoning (Blackburn BG et al., 2004). Moreover, a recent EFSA report accounts tap and well water as the source of 5% of the total reported outbreaks of gastroenteritis due to calicivirus (including NoVs). Even if these percentages might appear less relevant than other sources of contaminations, the same data analyzed in terms of mean number of human cases per outbreak reveal that water-borne outbreaks caused by caliciviruses (including NoVs) are the second cause of infection. In other words, water-borne outbreaks caused by viruses seem to represent a small fraction of the total reported cases, but can result in bigger outbreaks compared to other causes of contamination. Moreover, irrigation water has been identified as potential source of primary contamination for fruits and vegetables (FAO, 2008).
Several outbreaks of NoV gastroenteritis have been associated to the consumption of contaminated drinking water (Werber D et al., 2009; Scarcella C et al., 2009; O'Reilly CE et al., 2007; Boccia D et al., 2002; Kukkula M et al., 1999; Kukkula M et al., 1997;) or contaminated recreational water (Silva AM et al., 2009; Podewils LJ et al., 2007; Hoebe CJ et al., 2004; Kappus KD et al.,1982; Baron RC et al., 1982).

Since effective methods for water-borne virus recovery and detection are still on development and the current methods reported are often expensive, time-consuming and requiring cumbersome operations to be performed, the monitoring of viruses in water on routine bases appears to be beyond the capability of many private and public organizations responsible for drinking-water safety. Moreover, the absence of standardization and harmonization procedures complicates the translation of the scientific knowledge into a regulatory framework. It is currently well accepted that the only use of bacterial indicators, such as *E. coli,* for the assessment of microbial water quality, represents a not reliable index (WHO, 2008). However, from a risk management point of view, most of the guidelines or standard requirements include only qualitative considerations about the presence of viruses. When compliance requirements concerning virus in water are included in guidelines or standards,

they are usually defined in terms of virus occurrence or efficiency of the treatment for plant removal. These values result from the evaluation of the acceptable level of health risk or are strictly related to technical constrains, in terms of virus detection capability. In the last few years standard reccommendations and guidelines started progressively to include risk-based evaluation of water treatment requirements. The United States Environmental Protection Agency (USEPA) Groundwater Rule (USEPA, 2006) requires treatments that reliably achieves at least 99.99 percent (4-log) treatment of viruses (using inactivation, removal, or a State-approved combination of 4-log virus inactivation and removal) and include the needs of regular compliance monitoring to ensure the efficacy of the installed treatment technology. The groundwater rule was published in 2006 and took effect in December 2009. The WHO guidelines for drinking-water quality (WHO, 2008), which include a wide range of viruses as potential contaminants, state that water treatment requirements differ among different communities and identifies a risk-based strategy approach, taking into account a wide range of factors, such as virus occurrence and infectivity, community characteristics and water type. The need to develop a guidance on the control of viruses in food has also been recently stated within the Codex Alimentarius Commission: among the new defined works, the development of a general guidance document for the control of foodborne viruses is reported (ALINORM 09/32/13). The document, that will be supplemental to the *Recommended International Code of Practice – General principles of Food Hygiene* (CAC/RCP 1-1969, Rev. 4-2003), will also include a series of annexes to address the specific virus-commodity combinations, as prioritized by the FAO/WHO Expert Meeting on viruses in food: interestingly, NoVs and hepatitis A virus (HAV) in fresh products is considered as one of the priority and the main contamination route is reported to be irrigation water and manure. This suggests that the role of water as vector for virus contaminations is progressively gaining importance at various regulation and policy levels.

As a direct consequence of the paucity of knowledge about NoVs in water, no official or recommended methods specific for water-borne NoV recovery/detection have already been defined. However some methods have been reported for other viruses: i) USEPA published methods for virus recovery using Virus Adsorption-Elution (Viradel) disc filter (EPA/600/4-84/013) and cartridge filter (EPA/600/4-84/013) for recovering viruses from sewages and effluents and a method for concentration and processing of waterborne viruses by positive charge 1MDS cartridge filters and organic flocculation (EPA/600/4-84/013); ii) among the analytical methods for assessing the microbiological safety and general cleanliness of foods, prepared by the canadian regulatory body (Evaluation Division, Bureau of Microbiological

Hazards, Food Directorate, Health Products and Food Branch) a protocol for the "concentration of hepatitis A virus and rotavirus in spring or mineral bottled water samples is included together with their detection by the reverse-transcriptase polymerase chain reaction" (OPFLP-04, 2007); iii) a detection method for enterovirus based on glass wool concentration and detection by cell cultures is reported among the water and sanitization standards in France (AFNOR XP T90- 451): the method is currently under revision and the publication of the new version is planned for September 2010 (AFNOR, website).

Another major limitation in adopting water- and food-borne virus detection methods for routine and screening purposes is that legislation requires to include virus controls in standardized methodologies: the European Committee for Standardization is currently working on the elaboration of 'horizontal methods for the molecular detection of viruses in food' (CEN/TC275/WG6/TAG4 working group) and included bottled water among the tested matrices (UK NRL discussion paper, 2008; FAO/WHO, 2008).

In addition to the needs for international rules, guideline recommendations and harmonized standards, the creation of network databases and rapid monitoring systems collecting information about outbreaks and contaminations is essential, especially for emerging pathogens like NoVs. Examples of these systems are the European Community's Rapid Alert System for Food and Feed (RASTAFF) defined in the implementation of the European Community regulation 178/2002/EC (Kleter GA et al., 2009), the International Food Safety Authorities Network (INFOSAN) hosted by the World Health Organization (WHO) and its computerized Global Outbreak Alert and Response Network (GORAN) (Kleter GA and Marvin HJP, 2009) and, concerning the US, FoodNet, EFORS, CaliciNet, the Foodborne Outbreak Response Unit and PulseNet (Marvin HJP et al., 2009).

Concerning NoVs in particular, the Food-borne Virus in Europe (FBVE) network database plays an important role in monitoring trends in outbreaks of gastroenteritis due to NoVs, identifying major transmission routes of infections within and between participating countries and detecting international food-borne outbreaks (Kroneman A et al., 2007).

The concentration of viruses in water, including NoVs, is usually too low to allow the direct detection (excluding some cases such as sewage, where the presence of viruses may be sufficient to be directly detected). To overcome this limitation, several approaches, often involving multi-step concentration procedures, have been developed.

According to several reports and documents published (Wyn-Jones P, 2007; Bosch A et al., 2008), an ideal method of concentration should fulfil several defined requirements:

i) to be able to concentrate a wide range of viruses;
ii) to provide a small volume at the end of the process;
iii) to be able to process different volumes and types of water;
iv) to be repeatable and reproducible;
v) to be technically easy;
vi) to requires short time;
vii) to be not costly;

According to the same authors, no single method available at the moment meets all these requirements. The choice for the appropriate approach and the most suitable method strongly depends on the experimental purposes (type and volumes of water to be filtered, type of pathogens, method of detection after concentration) and the experience and expertise of the user might play a role in the selection process for the most appropriate system (Bosch A et al., 2008). Despite the applications of recovery strategies for water-borne viruses have been investigated since the late 1960s, the majority of the methods are still at a developmental stage and are used mainly in research monitoring than as routine diagnostic systems. This because, regardless of the recovery efficiency, they are not optimized for "end-user applications", are often expensive and require cumbersome procedures.

The most common methods for water-borne virus recovery described in book chapters, reviews and reports (Wyn-Jones P, 2007; Bosch A et al., 2008; Affset, 2009), along with their main advantages and disadvantages, are reported in Table 4.1.

The methods mostly used for concentration are commonly based on three steps: adsorption, elution and subsequent secondary concentration. The adsorption phase is performed using a wide range of materials and the recovery efficiency depend on several parameters such as the type of virus, the type of water (clean or turbid), its chemico-physical profile (pH, salts, types solid particles present in the sample), the sample volume and the filtration rate.

The availability of a wide range of membranes with various dimensions, composition and pore size made the membrane-based adsorption-elution methods extremely common for water-borne virus analysis.

Table3.1. Methods for virus concentration from water

Method	Principle	Pros	Cons
Adsorption-Elution Methods	Interations based on charge		
Positively charged filters		- Good recoveries - Wide range of volumes	-Costly
Negatively charged filters		- Good recoveries - Wide range of volumes	- Require sample preconditioning (difficult in-line and field applications) -Problems with viruses that not tolerate low pH
Glass powder		-Cheap -Good recoveries	-Need for a special, fragile apparatus
Glass Wool		-Extermely cheap -Good recoveries -Less clogging problems than membrane-based filters/cartridges -No sample pretreatments required (applicable for in-line and fields applications)	-Variability (differences depending on manufacturers and filter procedures)
Ultrafiltration-based methods	Particle size separation	-Very good recovery (clean samples) -Reusable filters	-Costly -Time-consuming -Filter sanitization require cumbersome procedures -Filters prone to clog
Ultracentrifugation	Physical separation (sedimentation)	-Efficient for secondary concentration	-Costly -Small volumes -Need for special apparatus
Precipitation-based methods	Chemical interactions		
Organic flocculation		-Efficient for secondary concentration or with dirty samples	- PCR/RT-PCR inhibition problems (beef extract) -Efficiency varies upon the virus type
Polyethylene glycol		-Efficient for secondary concentration or with dirty samples	-PCR/RT-PCR inhibition problems -Different recovery depening on the employed elution buffer
Ammonium sulfate		-Efficient for secondary concentration or with dirty samples	High cytotoxicity
Magnetic beads/Immunocapture	Immunoaffinity	-Good recovery	-Small volumes -Requires a specific system for each virus -Costly -Poorly investigated (little data available)

Negatively charged filters made of cellulose acetate or nitrate and negatively charged membranes constituted by cellulose/diatomaceous earth/ion-exchange resin mixtures or charged modified glass/cellulose media, are among the most commonly used materials (Wyn-Jones P, 2007). Recently new filter formulations, such as membranes based on nanoallumina dispersed throughout microglass fiber matrices , have been proposed as effective alternatives for virus filtration.

One of the major limiting step affecting the adsorption-elution methods is the procedure performed to remove (elute) the viruses from the filter; this operation is often combined with a secondary concentration procedure, in order to obtain suitable volumes for the subsequent (molecular or cell culture-based) analysis. These methods have some limitations and major drawbacks: i) the elution is commonly carried out using protein rich buffers (such as 1-3% of beef extract) at alcaline pH (9-11). The beef extract is suspected to have an inhibitory effect on the enzymes involved in the reverse transcription (RT) and in the polymerase chain reaction (PCR) procedures (Abbaszadegan M et al.,1993). In order to avoid buffers that contain proteins like the beef extract, other procedures have been proposed, such as a double-step approach based on membrane treatment with H_2SO_4 followed by elution with NaOH (pH 10.8) (Katayama H et al., 2002; Haramoto E et al., 2004). However, all these procedures are performed using alcaline conditions and require tempestive pH adjustment in order to avoid virus damages or inactivation. Also the procedures based on PEG have been identified as source of virus losses and RT-PCR inhibition, with a variable impact depending on the elution buffer employed and the type of virus eluted (Hamza IA et al., 2009). Moreover, the majority of these methods require long procedures (from several hours to overnight steps) and involve cumbersome operations with numerous manual steps, making difficult the development of any semi- or full-automated approach.

4.2 MATERIALS AND METHODS

Virus stock. Norovirus samples belonging to the genogroup II were obtained from stool specimens. The stool was diluted 1/10 (w/v) using molecular biology grade water and then centrifuged in order to remove the particulate matter. The aqueous phase was collected and stored at -80 °C.

The NoVs strains used were not sequenced and no informations about the genotype were collected.

Glass Wool types. Four different glass wools were selected for preliminary evaluations in terms of compressibility and chemical stability, using the same buffers and chemicals employed during the elution process (Internal Report for details). Operative flow rates and couter-pressure were also taken into account and roughly evaluated pumping small volumes of water (50 ml) with a syringe through the columns packed with the selected types of glass wool.

Glass Wool Column Preparation. Glass wool filters were prepared according to previously described procedures (Vilagines P et al, 1993; UK Environment Agency, 2000; Lambertini E et al., 2008) with minor modifications. Different types of glass wool were rinsed with MilliQ water for 15 min, washed with (1M) HCl for 15 min, rinsed again with water, washed with (1M) NaOH for 15 min and rinsed thoroughly with water until the pH was close to the neutrality (pH 7.0). The treated glass wool was used immediately or stored in sterile phosphate-buffered saline (PBS) solution, pH 7.2) at 4 °C for no longer than two days.
The glass wool was packed into a column using a sterile syringe plunger to a final density of 0.5 g/cm^3. In order to assure a constant density, the columns were packed in small fractions. The column size used in the experiments was 16 mm-diameter by 4.5 cm. The columns were polyethylene drying tubes (SIGMA-ALDRICH CHEMIE GmbH, Germany) capped with polypropylene end fittings provided with the tubes.

Spiking experiments. In order to characterize the glass wool-based filters, small volumes (50-100 ml) of deionized (milliQ) or commercial bottled water were spiked with a known amount of clarified stool, previously tested as positive for NoV GII. The performances of the filters were then evaluated using 1L of water. The bottled mineral water was purchased in a local store. Various brands were selected for the evaluation, in order to assess the effect of the pH and the salts composition on the efficiency of recovery. Small volumes of water (50-100 ml) were passed through the filters using sterile syringes, whereas larger volumes were pumped by means of a peristaltic pump (DOSE IT P910, INTEGRA Biosciences) using 4 mm-diameter silicon tubing at flow rates of 50-55 ml/min.

Virus elution. The elution of viruses from the glass wool filter and the subsequent processation steps were carried out according to the internally established protocol (confidential informations). Since the details of the elution process and the chemical

composition of the elution buffers can not be disclosed, the block of operations required to elute the viruses from the glass wool filter and the subsequent steps needed for the further processation of the eluate, will be referred in the rest of this document as the "elution process", "elution procedure" or "elution phase"; similarly, the mix of chemical substances employed in the elution process will be referred as "elution buffer".

Nucleic acids extraction. The eluates collected from the filtration experiments were processed (confidential informations) and the nucleic acids were extracted and purified using the NucliSens® easyMAG™ platform (bioMérieux, Marcy l'Etoile, France). The conventional 'on-board' or 'off-board' protocols provided with the commercial software were used, depending on the experiments. Nucleic acids were eluted with 40 μl elution buffer.

Real Time RT-PCR detection and virus quantification. Internally developed Single-tube real time RT-PCR optimized for NoV GII detection was used to analyze the samples filtrated, according to the internal protocols (Internal Reports for details and reaction conditions). All the assays were performed in a 50 μl reaction mixture using an MX3000P QPCR system (Stratagene, La Jolla, CA, USA). For each PCR run, 10-fold dilutions of internally developed calibration materials, originated from synthetic oligonucleotides, were used to calculate a standard curve (Internal Report for details). All the calibration materials underwent an internal quality control and validation process involving quantification with spectrophotometric techniques and characterization using Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Each serial dilution was assessed in duplicate or triplicate.

4.3 RESULTS AND DISCUSSION

The use of glass wool-based filters for water-borne virus concentration has been reported as an effective strategy in several scientific publications and it has also been included in official guidelines and reports (Lambertini E et al., 2008; Vilagines P et al.,1993; AFNOR XP T90-451). However, the performances of glass wool-based filters have not yet been sistematically investigated for NoV recovery. Moreover, the mechanism that rules the interactions between the capsid and glass wool fibers has not been completely clarified: the majority of the

applications reported using the glass wool as virus trap, were carried out applying the same protocols (with minor modifications) described in the first published papers. Consequently, the feasibility of a glass wool-based device for NoV recovery form water were investigated, attempting to achieve a major improvement; the following major concerns were taken into account during the design phase:

i) Costs: the filtering device should be inexpensive, in order to meet the needs for NoV analysis in water. Morover, even if it should be easily integrated in a semi- or full- automated system, it should not imperatively require expensive instrumentation to be succesfully employed as filtration system. The development of effective methods for water-borne viruses detection requires low costs to be routinely employed in both industrial and environmental settings: the mild nature of numerous water-borne virus infections and the current lack of precise norms do not justify the need for expensive detection methods.

ii) Automation: the filtering device should be user friendly and not requiring numerous manual operations. Moreover it should be easily integrated in an automated system, in order to meet the needs for med- and high throughput solutions. The majority of methods based on adsorption-elution strategies described in the scientific literature and in the official reports, involve numerous subsequent manual operations which makes the process difficult to automatize.

iii) Time-to-results: the reported adsorption-elution methods require time-consuming elution and further secondary concentration processes, with steps ranging from hours to over-night treatments. Moreover, these processes usually require the presence of an operator to be performed and the required time for the overall process (filtration-elution-nucleic acids extraction and viruses detection) easily exceed one working day. The ideal method should aim to optimize the process, in order to shorten and simplify the overall procedure.

iv) Scalability and modularity: the filtering device should be easily employed for filtering a wide range of volumes and types of water (from clean bottled water to dirty water) with adeguate flow-rates and no clogging problems. If one filtering device results insufficient in certain conditions, a combination of several device units should be sufficient to overcome the major limitations.

According to the defined major concerns and after a survey about the available virus filtration methods, a glass wool-based filtration device appeared to be a balanced compromise among all the considered parameters. Glass wool filters seem to: i) be extremely inexpensive; ii) assure good flow-rates with different types of water; iii) be less prone to clog than membrane-based filter; iv) do not require expensive equipment to perform the analysis; v) be more efficient in trap virus capsids than naked nucleic acids, thus reducing the risks of detect non-infectious viruses using molecular methods; vi) be effective for the recovery of a wide range of viruses, allowing multiple analyses for screening purposes; vii) do not require any sample pre-treatment step, opening the possibility to use glass wool-based devices for *in-line* (industrial settings) or *in-the-field* (environmental settings) analyses. The only current major drawback in glass wool-based water-borne viruses filtration seems to be the high variability affecting the overall process (recovery-elution-secondary concentration-detection). No evident reasons for this extreme variability have been already reported, but different types of glass wool and the procedure employed to pack the filters are suspected to be the source of variability (Wyn-Jonrs P, 2007; Lambertini E et al., 2008). Moreover, due to the lack of efficient cell culture methods to cultivate and analyze NoVs, few reports include this virus in the glass wool filtration experiments. On the other hand it must be noticed that no systematic and comprehensive studies on glass wool-based filtration methods have been reported. This work takes into account the previously described details, in order to evaluate the feasibility of a glass wool filter for NoV recovery from water with the major aims to: i) investigate the glass wool filter preparation, ii) simplify and optimize as much as possible the elution/secondary concentration process, iii) obtain preliminary results to evaluate the overall filtration-detection system with different types of water.

The size of the columns used during the feasibility study was defined according to:

i) previous published results (UK Environment Agency report, 2000), according to which the column size suitable for the effective virus recovery is 16mm diameter by 6.6 cm; according to this report, an increased diameter or a longer column do not further improve the performances of the method;

ii) an internal optimized design for the elution process: the column length was furhter reduced to 4.5-5.0 cm.

The density of the packed glass wool into the columns was kept constant at 0.5 g/cm^3 as previously reported (Vilagines P et al., 1993; Lambertini E et al., 2008). The 0.5 g/cm^3 density seems to be approximately the maximum obtainable density for the used glass

wool, without affecting (disrupt) the fibers structure (internal observations). Particular attention has been payed to the packing procedure, since the density seems to be one of the most important parameter in order to have an effective virus recovery (UK Environment Agency, 2000).

The procedure concerning the elution/secondary concentration process (details can not be disclosed in this report) was designed in order to:

i) reduce the total number of manual operations and shorten the time required to perform the whole procedure, in order to provide a user-friendly workflow and reduce virus losses due to multiple-steps operations;

ii) avoid as much as possible the employment of substances known to be source of high variability or substances that play inhibition effects on the enzymes used in molecular-based detection methods, such as the DNA polymerases. For example, beef extract-based solutions are widely employed as elution buffers. However these kind of buffers are a well known source of PCR inhibitors. Moreover, beef-extract seems to be affected by high batch-to-batch variability and it has been suggested in official reports (UK Environment Agency, 2000) that each batch should be tested as suitable for elution purposes. This drawbacks might complicate the transfer and the scaling of these solutions from a research context to an industrial level. Also the secondary concentration procedures based on PEG precipitation seems to be affected by high variability, depending on the elution conditions employed: according to a recent publication (Hamza IA et al., 2009) the detection efficiency of virus nucleic acids resulted to be reduced using PEG; moreover, the magnitude of this effect change, depending on the used elution buffer. In particular, the efficiency in detecting NoV GI samples, using a combination of beef extract-based elution buffer and PEG precipitation, turned out decreased to 10% of the initial virus amount (Hamza IA et al., 2009);

iii) avoid the use of substances that can provide chemical or additional biological risks for the operators.

Concerning the types and volumes of water to be tested during the feasibility study phase, the samples to be investigated were:

i) bottled water (1.0-1.5 L). Bottles of mineral water were purchased in local markets and used for the filtration experiments. Bottled water was selected for several

reasons: 1) the well-defined salt composition and chemico-physical parameters provide a sort of "standardized matrix" that reduces the risks of variability due to the type of water and allow to correlate the efficiency of viruses recovery with the known water parameters; 2) the small volumes and the low turbidity make easier the optimization of the filtration procedure during the initial stages; 3) bottled water is included among the food matrices (along with soft fruits, leafy greens, hard surfaces and shellfish) that are currently being validated by the European Committee for Standardization (CEN) technical commitee CEN/TC 275 in the working group WG6 TAG4 (FAO/WHO, 2008; UK NRL discussion paper, 2008);

ii) well and river water as types of naturally contaminated water;

iii) irrigation water as type of water involved in food contaminations.

Since only one type of glass wool was used in each reports and published papers regardless of the chemical composition and no comparisons among different types and brands of glass wool have currently been reported, the first objective of this project, was to compare different types of glass wool for the recovery of NoVs from water, as described in the Materials and Methods Section. The types of glass wool evaluated in this work with their important characteristics are reported in Table 4.2. All the selected types of glass wool were tested for their chemical stability covering 2 g of each fiber type with the same chemical substances used during the whole process. All the types of glass wool showed to be stable when kept in contact with the chemical substances for more than two hours. The four types of glass wool were then packed in columns and evaluated as described in the Materials and Methods Section. Some differences were observed among the different types of glass wool in terms of maximum packing density (between 0.3 g/cm^3 to 0.5-0.6 g/cm^3) and in terms of flow rates (from 0.1 L/min to 0.6 L/min) and counter-pressures. According to these observations, two types of glass wool were selected for further evaluations, filtering small amounts of water artificially contaminated with NoVs.

Table 4.2. Types and main characteristics of the glass wool evaluated in this study

	Glass wool 1	**Glass wool 2**	**Glass wool 3**	**Glass wool 4**
Main uses	Thermal insulation for industrial equipments (for example industrial owens)	Thermal insulation for walls, roofs and panels	Special purposes	Special purposes
Chemical Composition				
-Silicon dioxide (silica) (SiO_2)	63-66%	Not disclosed	65.5%	58.3%
-Titanium oxide (Ti_2O_3)	2-4%		/	/
-Iron (III) oxide (Fe_2O_3)	0-1%		/	/
-Boron trioxide (B_2O_3)	4-7%		5.3%	11.3%
-Calcium oxide (CaO) + Magnesium oxide (MgO)	7-12%		5.9% + 3.0%	1.8% + 0.3%
-Sodium (Na_2) + Potassium monoxide (K_2O)	15-19%		16.0% + 0.7%	10.1% + 2.9%
-Aluminium Oxide (Al_2O_3)	/		3.1%	5.8%
-Barium Oxide (BaO)			0.01%	5.0%
-Zinc Oxide (ZnO)			/	4.0%
Relevant MSDS informations	-Classified as skin irritant (R38) -No phenolic resins -Not classified as carcinogenic material according to the European directive (97/69/CE)	Not found	-Classified as respirable size fibers - Potential human carcinogenic	-Classified as respirable size fibers - Potential human carcinogenic
Use of formaldehyde	No	No	Not reported in the MSDS sheet	Not reported in the MSDS sheet
Previously reported use as filtration media for virus or cells	Yes [(a)] (virus filtration)	No	No	Yes [(b)] (sperm filter trap)
Measured performances (max density, flow rates, counter-pessure)	0.5-0.6 g/cm^3; 0.6 L/min; low	0.5-0.6 g/cm^3; 0.1-0.2 L/min; high	0.2-0.3 g/cm^3; too low; too high	0.2-0.3 g/cm^3; too low; too high

Notes: (a). Vilagines P et al., 1993; Lambertini E et al., 2009; (b). Cary JA et al., 2004; Jeyendran RS, US Patent;

Since the elution process was defined *ex novo* during the design phase and no previous information has been found in the scientific literature concerning analogous approaches, the first experiment aimed to assess the feasibility of the designed elution process coupled with NoV recovery from water using glass wool filtration. The estimation of the virus recovery and the evaluation of the best elution procedure were performed using 21 ml of deionized water spiked with a high NoV titre (3 x 10^6) in order to enable the estimation of the viral concentration in the water, before and after the filtration process. The sample was thoroughly mixed to assure a homogeneus virus concentration and an aliquote (1 mL) was immediately collected and used to estimate the initial virus concentration. Two aliquots of 10 mL each were then filtered using two columns previously prepared with the same stock of pretreated glass wool (Glass wool 1). Two protocols of the same elution process were evaluated ("elution a" and "elution b"). According to the results (Table 4.3) a consistent fraction of the total spiked virus amount (around 40%) is detectable in the water after the filtration process.

The reasons for this high fration of not absorbed virus might be correlated with: i) the high virus titre: a too elevated rate of virus particles x ml^{-1} x sec^{-1} might results in a decreased efficiency of recovery; ii) the type of water used: according to previous reported evidences the efficiency of recovery decreases when salt-depleted water is used (Vilagines P et al., 1988). Since the "elution a" protocol showed higher recovery, it was chosen as elution procedure in the other experiments.

Table 4.3. Evaluation of NoVs recovery from water using glass wool-based filtration and two elution protocols using the internal defined elution process

	% Recovery [(a)]	
	Elution process	**Filtered water**
Column 1 (elution a)	45.1	43.6
Column 2 (elution b)	19.6	36.2

Note: (a) All the calculations were performed using total detectable genome copies derived using standard curve values.

In order to compare the performances of the two types of glass wool selected during the evaluation phase, one column for each type of glass fibers were prepared and used for the filtration of 55 mL of deionized water artificially spiked with NoVs (Table 4).

Table 4.4. Evaluation of the selected types of glass wool for NoVs recovery

	Spiked [(a)]	**% Recovery (Elution process)**	**% Recovery (Filtered water)**
Glass wool 1	1.6×10^6	26.5	50.0
Glass wool 2	1.5×10^6	20.4	67.3

Note: (a) detectable genome copies spiked in 55 mL

The results showed similar recovery rates; however, since the Glass wool 1 was found to allow broader range of operative flow-rates with less counter-pressure than the Glass wool 2, the following experiments were carried out employing the Glass wool 1 as adsorption material. During these first experiments, the column preparation procedure and the parameters for the elution process were also optimized, in order to minimize as much as possible the contribution of the manual operations to the overall process variability. For this reason some minor modifications were introduced in the following experiments, the elution step duration and the procedure by which the elution buffer is dispensed in the colums were defined more precisely.

The previously described experiments were then repeated with a different type of water (commercial bottled mineral water) using two different volumes (55 mL and 1 L), attempting to assess the contribution of different volumes of filtered water on the efficiency of recovery (Table 4.5). According to the results, the increased sample volume did not negatively affect the recovery rate using the optimized filtration-elution method.

Table 4.5. Evaluation of the selected types of glass wool for NoV recovery

	Spiked [(a)]	**Filtration rate (mL min^{-1})**	**% Recovery (Elution process)**
Bottled 55mL	2.3 x 10^6	55 (manually)	47.1
Bottled 1L	1.1 x 10^6	55 (peristaltic pump)	41.6

Note: (a) detectable genome copies calculated for the same amount of virus spiked in water (5 uL) using the same clarified stool aliquot

The effect of the chemico-physical characteristics and the variability of the overall process were further evaluated testing three different brands of commercial mineral water and well water (Table 4.6). Each analysis were performed in triplicate or duplicate. The brands of bottled water were selected in order to have different pH (from 5.85 to 8.00) and different salt concentrations. The glass wool filters were effective in concentrating NoV from the three brands of bottled water; however the recovery differed among water types. The brand 1 and brand 3 showed to have the highest recovery rates (respectively 98% and 74%) which can be

considered similar taking into account the variability among different trials (respectively 26% and 30%).

Table 4.6. Comparison of NoV recovery from different types of water and different virus concentrations.

Water type	No of trials [(a)]	Tot gen copies seeded	% Mean Recovery (SD)
Bottled (brand 1)	3	1.39×10^5	98 (±26)
Bottled (brand 2)	3	1.46×10^5	37 (±13)
Bottled (brand 3)	3	1.71×10^5	74 (±30)
Bottled (brand 3)	2	6.33×10^3	> 100 (±14)
Bottled (brand 3)	2	6.90×10^2	40 (±14)
Well	2	7.53×10^4	65 (±8)

Note: (a) all tests were performed using a peristaltic pump with a filtration rate of 55 mL min^{-1}

Interestingly, the lower recovery was found to be related to the water brand containing the lowest amount of salts (Table 4.7) thus supporting the evidence that low amount of salts (especially divalent cations) might play an important role in the interactions between viruses and glass wool (Vilagines P et al., 1988). Moreover, comparing the recovery from brand 1 and brand 2, the presence of salts resulted to be more important than pH; this observation is quite surprising because the pH was defined as one of the most critical parameter for an effective virus recovery and, generally speaking, low values of pH seem to increase the virus recovery, although with different contributions depending on the virus characteristics (such as the isoelectric point and the aminoacidic composition of the capsid).

However, these results seem to be in agreement with previous observations showing that the variability of virus recovery as function of pH result mitigated by the presence of salts (Vilagines P et al.,1988). However, even if some general correlation can be outlined, it is difficult to infer general behaviours and rules comparing these experiments with the previously published reports, since all the experiments were carried out using different experimental conditions and different virus types and strains.

Table 4.7. Chemico-physical analyses reported on the bottle of the different brands of bottled water used in the experiments.

Parameters	Data	Water 1	Water 2	Water 3
Acidity	pH	8.00	5.85	7.20
Cations (mg/L)	Calcium	41.00	2.40	80.00
	Magnesium	3.00	0.50	26.00
	Sodium	2.00	3.10	6.50
	Potassium	0.00	0.40	1.00
Anions (mg/L)	Bicarbonate	134.00	6.30	360.00
	Chloride	3.00	3.00	6.80
	Nitrates	3.00	3.00	3.70
	Sulphates	2.00	2.00	12.60
Other informations (mg/L)	Fluoride	< 0,05	< 0,1	/
	Silica	/	8.20	15.00
	Residue fixed (180°C)	139.00	25.00	309.00

Note: The most representative data are reported in red

In order to better define the elution procedure, the eluates were collected in three subsequent fractions and separately analyzed (the recovery rates reported in Table 4.6 represent the total contributions of the three fractions). The recoveries of the single fraction supposed to contain the majority of the concentrated viruses, according to the column design are reported in Figure 4.1. As expected, more than the 80% of the totally viruses recovered are collected in a single fraction. According to these data, it seems possible to design and further optimize an elution method using a single fraction of elution buffer. This might help to avoid virus losses and simplify the design of the whole filtration process.

The recovery of NoV with more diluted samples was assessed spiking a serially diluted amount of clarified stool using bottled water, brand 3 (the type of water that gave the mean average recovery among the used bottled waters). The less concentrated sample tested (0.69 genome copies L^{-1}) resulted in a mean recovery of 40%; these results can be considered as satisfatory compared with the previously published data where higher viruses titres are usually employed. Moreover, the concentration from well water resulted in a good recovery rate (around 65%), showing the applicability of this method for different types of water, including environmental samples. This might be very useful for screening purposes since well water is known to be a source of virus, usually due to accidental wastewater contaminations.

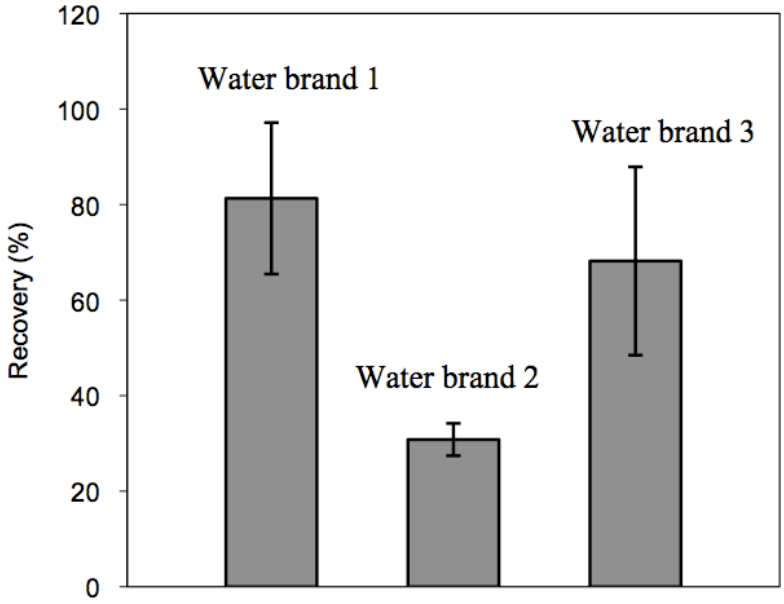


Figure 4.1. Recovery for the single fraction (out of three) supposed to contain the majority of the recovered viruses according to the design

However, it must noticed that in order to be effective, the method here developed must be scalable to larger volumes of water and must tollerate higher filtration rates without dramatically decrease the recovery performances. These evaluations will be included in future experiments.

The possibility to filter turbid fluids using the glass wool filters were tested filtering 1L of orange juice with pulp residues. The filters worked perfectly using a flow rate of 55 mL min^{-1} and never clogged; however, applying the same procedure optimized for water filtration, the virus recovery resulted to be very low (less than 1%). However, this result might open the possibility to adapt the same filters to the filtration of liquid foods designing optimized procedures.

The last technical aspect investigated during the feasibility stage was the potential reduction on the EasyMag extraction efficiency due to the occasional presence of small fibers of glass wool during the extraction process. This effect was assessed adding a known amount of glass wool to the extraction mix (from 20 seconds to 15 minutes). No efficiency reduction effects on the extraction and purification procedure were observed (Figure 4.2). However, the column design will be optimized in the next experiments in order to eliminate the losses of small fibers.

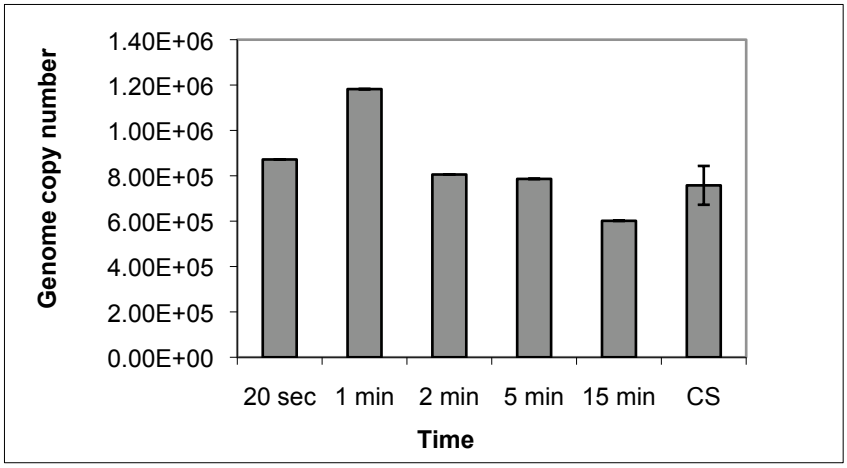


Figure 4.2: Effect of the presence of glass wool on the nucleic acid extraction procedure. CS= clarified stool (reference value).

According to the results obtained, the glass wool-based filters coupled with the new designed elution process provide an easy method to concentrate NoVs from water with good recovery rates, comparable or even better than the previously reported methods. The major advantages of this method, compared with the previously reported approaches are:

i) shorter time-to-results: the total time required for the elution and the nucleic acid extraction/purification does not exceed the 60 minutes, thus reducing the overall time for the analysis. The elution phase is currently one of the limiting step in terms of time and might represent the bottleneck of the overall analysis process in terms of time and number of manual operations.

ii) More user-friendly procedure: the number of manual operations are dramatically reduced and the overall process requires easier steps to be performed.

iii) High automation potential: the reduced number of manual operations, allows the design of a semi- or full- automated strategy for an integrated virus concentration/detection approach.

iv) Reduced costs: the glass wool based filter is extremely inexpensive and does not impact on the overall analysis costs.

On account of these characteristics, the method developed might represent a promising tool for the analysis of water-borne viruses. However numerous aspects must be further investigated in order to consider the method described for this purpose.

4.4 REFERENCES

Abbaszadegan M, Huber MS, Gerba CP, Pepper IL (1993). Detection of enteroviruses in groundwater with the polymerase chain reaction. Appl Environ Microbiol;59(5):1318-24.

AFNOR XP T90- 451, standard: Testing water - Detection of enterovirus - Method by concentration on glass wool and detection by cell culture

AFNOR, website: http://www.afnor.org/en/sectors/activity-area/water-and-sanitation/normes/normes-a-l-etude/%28domaine%29/24800

Affset, 2009: "Risques sanitaires liés à la présence du virus Influenza aviaire dans les eaux. Virus Influenza aviaires hautement pathogènes de sous-type H5N1 : stratégies d'échantillonnage dans les milieux aquatiques. Agence française de sécurité sanitaire de l'environnement et du travail, Avril 2009.

Baron RC, Murphy FD, Greenberg HB, Davis CE, Bregman DJ, Gary GW, Hughes JM, Schonberger LB (1982). Norwalk gastrointestinal illness: an outbreak associated with swimming in a recreational lake and secondary person-to-person transmission. Am J Epidemiol;115(2):163-72.

Blackburn BG, Craun GF, Yoder JS, Hill V, Calderon RL, Chen N, Lee SH, Levy DA, Beach MJ (2004). Surveillance for waterborne-disease outbreaks associated with drinking water--United States, 2001-2002. MMWR Surveill Summ;53(8):23-45

Boccia D, Tozzi AE, Cotter B, Rizzo C, Russo T, Buttinelli G, Caprioli A, Marziano ML, Ruggeri FM (2002). Waterborne outbreak of Norwalk-like virus gastroenteritis at a tourist resort, Italy. Emerg Infect Dis;8(6):563-8.

Bosch A, Guix S, Sano D, Pintó RM (2008). New tools for the study and direct surveillance of viral pathogens in water. Curr Opin Biotechnol;19(3):295-301.

Centers for Disease Control and Prevention (CDC) (2009). Surveillance for foodborne disease outbreaks - United States, 2006. MMWR Morb Mortal Wkly Rep;58(22):609-15

EFSA 2009: The Community Summary Report on Food-borne Outbreaks in the European Union in 2007, The EFSA Journal (2009), 271).

EPA/600/4-84/013: "USEPA Manual of Methods for Virology"

FAO/WHO, 2008: Food and Agriculture Organization of the United Nations/World Health Organization. "Viruses in Food: scientific advice to support risk management activities". Microbiological Risk Assessment Series No. 13, 2008. Rome.

Hamza IA, Jurzik L, Stang A, Sure K, Uberla K, Wilhelm M (2009). Detection of human viruses in rivers of a densly-populated area in Germany using a virus adsorption elution method optimized for PCR analyses. Water Res;43(10):2657-68.

Haramoto E, Katayama H, Ohgaki S (2004). Detection of noroviruses in tap water in Japan by means of a new method for concentrating enteric viruses in large volumes of freshwater. Appl Environ Microbiol;70(4):2154-60.

Hoebe CJ, Vennema H, de Roda Husman AM, van Duynhoven YT (2004). Norovirus outbreak among primary schoolchildren who had played in a recreational water fountain. J Infect Dis;189(4):699-705.

Jeyendran RS (1996). Sperm filter trap having compressed glass wool filter material, US Patent 5575914. November 19, 1996

Kappus KD, Marks JS, Holman RC, Bryant JK, Baker C, Gary GW, Greenberg HB (1982). An outbreak of Norwalk gastroenteritis associated with swimming in a pool and secondary person-to-person transmission. Am J Epidemiol;116(5):834-9.

Katayama H, Shimasaki A, Ohgaki S (2002). Development of a virus concentration method and its application to detection of enterovirus and norwalk virus from coastal seawater. Appl Environ Microbiol;68(3):1033-9.

Kleter GA, Marvin HJ (2009). Indicators of emerging hazards and risks to food safety. Food Chem Toxicol;47(5):1022-39.

Kleter GA, Prandini A, Filippi L, Marvin HJ (2009). Identification of potentially emerging food safety issues by analysis of reports published by the European Community's Rapid Alert System for Food and Feed (RASFF) during a four-year period. Food Chem Toxicol;47(5):932-50.

Kroneman A, Harris J, Vennema H, Duizer E, van Duynhoven Y, Gray J, Iturriza M, Böttiger B, Falkenhorst G, Johnsen C, von Bonsdorff CH, Maunula L, Kuusi M, Pothier P, Gallay A, Schreier E, Koch J, Szücs G, Reuter G, Krisztalovics K, Lynch M, McKeown P, Foley B, Coughlan S, Ruggeri FM, Di Bartolo I, Vainio K, Isakbaeva E, Poljsak-Prijatelj M, Grom AH, Bosch A, Buesa J, Fauquier AS, Hernandéz-Pezzi G, Hedlund KO, Koopmans M (2008). Data quality of 5 years of central norovirus outbreak reporting in the European Network for food-borne viruses. J Public Health;30(1):82-90.

Kukkula M, Arstila P, Klossner ML, Maunula L, Bonsdorff CH, Jaatinen P (1997). Waterborne outbreak of viral gastroenteritis. Scand J Infect Dis;29(4):415-8.

Kukkula M, Maunula L, Silvennoinen E, von Bonsdorff CH (1999). Outbreak of viral gastroenteritis due to drinking water contaminated by Norwalk-like viruses. J Infect Dis;180(6):1771-6.

Lambertini E, Spencer SK, Bertz PD, Loge FJ, Kieke BA, Borchardt MA (2008). Concentration of enteroviruses, adenoviruses, and noroviruses from drinking water by use of glass wool filters. Appl Environ Microbiol;74(10):2990-6.

Marvin HJ, Kleter GA, Prandini A, Dekkers S, Bolton DJ (2009). Early identification systems for emerging foodborne hazards. Food Chem Toxicol;47(5):915-26.

Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV (1999). Food-related illness and death in the United States. Emerg Infect Dis;5(5):607-25.

OPFLP-04, 2007: "Concentration of Hepatitis a Virus and Rotavirus in Spring or Mineral Bottled Water Samples and Their Detection by The Reverse-Transcriptase Polymerase Chain Reaction", 2007, Ottawa, Canada.

O'Reilly CE, Bowen AB, Perez NE, Sarisky JP, Shepherd CA, Miller MD, Hubbard BC, Herring M, Buchanan SD, Fitzgerald CC, Hill V, Arrowood MJ, Xiao LX, Hoekstra RM, Mintz ED, Lynch MF (2007); Outbreak Working Group. A waterborne outbreak of gastroenteritis with multiple etiologies among resort island visitors and residents: Ohio, 2004. Clin Infect Dis;44(4):506-12.

Patel MM, Hall AJ, Vinjé J, Parashar UD (2009). Noroviruses: a comprehensive review. J Clin Virol;44(1):1-8.

Patel MM, Widdowson MA, Glass RI, Akazawa K, Vinjé J, Parashar UD (2008). Systematic literature review of role of noroviruses in sporadic gastroenteritis. Emerg Infect Dis;14(8):1224-31.

Podewils LJ, Zanardi Blevins L, Hagenbuch M, Itani D, Burns A, Otto C, Blanton L, Adams S, Monroe SS, Beach MJ, Widdowson M. Outbreak of norovirus illness associated with a swimming pool. Epidemiol Infect. 2007 Jul;135(5):827-33.

Scarcella C, Carasi S, Cadoria F, Macchi L, Pavan A, Salamana M, Alborali GL, Losio MM, Boni P, Lavazza A, Seyler T (2009). An outbreak of viral gastroenteritis linked to municipal water supply, Lombardy, Italy, June 2009. Euro Surveill. Jul 23;14(29).

Silva AM, Vieira H, Martins N, Granja AT, Vale MJ, Vale FF (2009). Viral and bacterial contamination in recreational waters: a case study in the Lisbon bay area. J Appl Microbiol. Jul 29. [Epub ahead of print].

UK Environment Agency, 2000: "Optimisation of a new method for detection of viruses in groundwater National Groundwater and Contaminated Land Centre. Report no. NC/99/40, UK Environment Agency, July 2000.

UK NRL discussion paper 2008: UK National Reference laboratory (NRL) for monitoring bacterial and viral contamination of bivalve molluscs, Cefas, Weymouth, UK. June 2008 (v7)

Vilagines, P, Sarrette B, Husson G, Vilagines R (1993). Glass wool for virus concentration at ambient water pH level. Water Sci. Technol; 27:299–306.

Vilagines P, Sarrette B, Vilagines R (1988). [Continuous detection of poliovirus in public water supplies]. C R Acad Sci III.;307(4):171-6. French.

Werber D, Lausević D, Mugosa B, Vratnica Z, Ivanović-Nikolić L, Zizić L, Alexandre-Bird A, Fiore L, Ruggeri FM, Di Bartolo I, Battistone A, Gassilloud B, Perelle S, Nitzan Kaluski D, Kivi M, Andraghetti R, Pollock KG (2009). Massive outbreak of viral gastroenteritis associated with consumption of municipal drinking water in a European capital city. Epidemiol Infect;137(12):1713-20.

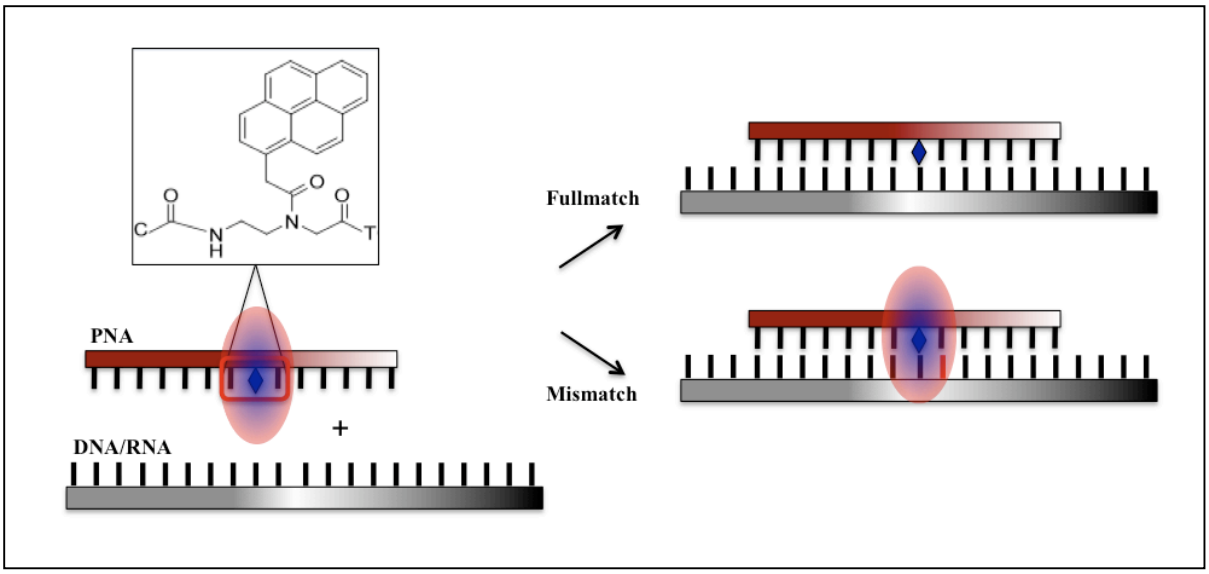
WHO, 2008: WHO Guidelines for Drinking water quality, 2008

Wyn-Jones P (2007): The detection of waterborne viruses. In Human Viruses in Water. Edited by Bosch A. The Netherlands: Amsterdam; 2007: 177-204.

Chapter 5

A pyrenyl-PNA probe for DNA and RNA recognition: fluorescence and absorption studies

With the general aim of developing new methods for norovirus detection we devised new PNA probes. To this purpose a PNA oligomer containing a pyrenyl residue in the backbone was designed and synthesized. The pyrenyl unit replaced a nucleobase in the middle of the PNA through covalent linking to the backbone by a carboxymethyl unit. A systematic study on the binding properties of this probe towards DNA and RNA complementary strands was carried out by UV and fluorescence spectroscopies.
Recognition experiments on mismatched DNA and RNA sequences were also performed. The combination of the two techniques allowed to obtain a clear discrimination of RNA against DNA and mismatch recognition, even using a "difficult" target such as a GC-rich sequence present in the genogroup II norovirus genome.



5.1 INTRODUCTION

With the general aim of developing new methods for norovirus detection we devised new PNA probes. To this purpose a PNA oligomer containing a pyrenyl residue in the backbone was designed and synthesized.

The development of novel, selective and sensitive sensor probes for DNA and RNA has become a very active research topic in the last few years, with applications in many different fields ranging from genetics to molecular biology and medical diagnostics. Beside recognition selectivity, nucleic acid detection also requires the use of very sensitive techniques for transducing the hybridization event in suitable detectable signals. Oligonucleotide probes modified with fluorophores are being extensively studied because of the high sensitivity attainable in nucleic acid recognition. In particular, pyrene-based dyes have been successfully used for nucleic acid labelling, due to their interesting photochemical properties, such as the long lifetime of the excited state, sensitivity to microenviromental changes and propensity to π-stacking (Winnik FM, 1993). In most cases the pyrenyl moieties were covalently linked to the nucleobase residues or to the phosphate chain at the terminus. Pyrene moieties have been appended at various positions in the sugar units of locked nucleic acids (LNAs) (Hrdlicka PJ et al., 2005; Kumar TS et al., 2008), altritol nucleotides and ribonucleotides in hexitol nucleic acids (HNA) (Wang G et al., 2009) and phosphoroamidite derivatives instead of a regular nucleobase (Paris PL et al., 1998). Moreover, direct conjugation at the backbone level has been reported for oligodeoxyribonucleotides (Christensen UB and Pedersen EB, 2002) and peptide nucleic acid (MacKinnon KF et al., 2007) and numerous works describing pyrene-conjugated nucleobases have also been reported such as the Py-G (Wanninger-Weiß C et al., 2008), ^{Py}U , ^{Py}C and ^{Py}A (Okamoto A et al., 2005), PydU (Mayer-Enthart and Wagenknecht HA, 2006) and 1-ethynylpyrene-modified guanine and cytosine (Wanger C et al., 2005). Moreover, other pyrene derivatives such as the ethynylpyrene (Mayer E et al., 2004) or silylated pyrene molecules have beeen employed for probe derivatization.

Pyrene-derivatized probes have been designed for several purposes such as detection of single nucleotide polymorphisms (SNPs) (Okamoto A et al., 2005; Masuko M et al., 1998; Paris PL et al., 1998), labelling of RNA poly(A) tracts (Tanaka K and Okamoto A, 2008) or probing conformational DNA duplex transitions (Okamoto A et al., 2005; Seo YJ and Kim BH, 2006). Different types of probes such as linear probes, Scorpion oligonucleotides (Gbaj A et al., 2008), dual probes-based systems (Masuko M et al., 1998; Paris PL et al., 1998) and molecular beacons (Mayer E et al., 2004; Conlon P et al., 2008; Yamana K et al., 2007) for

homogeneus hybridization assays and linear probes for microarray-based label-free detection of RNA (Sakamoto T et al., 2008) have been investigated. Moreover, several exciplex forming probes have been investigated (Bichenkova EV et al., 2005; 2006; 2007) as a strategy to provide detectors that match specific needs or allow for multiplexing.

At the same time, there is a remarkably growth in the use of synthetic oligonucleotide analogues in bioorganic chemistry, medicinal chemistry and molecular biology. Peptide Nucleic Acids (PNAs) suitable to be used for highly specific diagnostic systems, show very high affinity for complementary targets and very high selectivity, in particular for mismatch discrimination (Nielsen PE et al., 1991; Egholm M et al., 1993). On account of their hybridization efficiency and specificity, PNAs have been used in a wide range of diagnostic applications in connection with PCR, Real-Time PCR, Microarray, Fluorescence in situ Hybridization (FISH) and chromatographic techniques (Pellestor F and Paulasova P, 2006; Lundin KE et al., 2006; Brandt O and Hoheisel JD, 2004; Sahu N et al., 2008). Recently, fluorescent probes based on PNAs have been also reported: molecular beacons (Totsingan F et al., 2008), lightup probes (Lejion M et al., 2006) and FIT probes (Socher E et al., 2008). All these systems combine specific target recognition with fluorescence enhancement upon DNA or RNA complexation. One example of pyrenyl PNA has been reported, but with no data about its performance as fluorescent probe (Mackinnon KF et al., 2007).

Herein we describe the synthesis of a PNA oligomer containing a pyrenyl unit covalently linked to the backbone as a nucleobase substitute and its binding properties towards DNA and RNA complementary strands by UV and fluorescence spectroscopy. For the PNA sequence we chosed a sequence complementary to a gene tract of the genogroup II of norovirus which is particularly rich in GC. This kind of sequences are interesting because of the low mismatch discrimination efficiency obtainable with conventional DNA probe analysis. Thus, the discriminating ability towards mismatch inside the targeted region has been investigated.

5.2 MATERIALS AND METHODS

Synthesis of N_{α}-(N'-Boc-aminoethyl)glycine: H_2O (18 ml) and LiOH (0.83g) were added to a solution of Nα-(N'-Boc-aminoethyl)glycine ethyl ester in 30 ml of methanol. After 1 hour the reaction was completed. The methanol was then evaporated and the pH was adjusted to pH 7 by addition of a diluted HCl solution. The remaining water was evaporated under

vacuum and the product was obtained as a white solid. (yield. 50%). ^{1}H NMR (DMSO-d6, 300MHz): δ(ppm) 1.34 (s , 9H, CH3 Boc), 2.79-2.84(m, 2H, CH2 aminoethyl), 3.15-3.19(m, 4H, CH_2 aminoethyl + CH_2 glycine). ^{13}C NMR (DMSO-d6, 75MHz) δ(ppm): 28.03, 36.73, 46.75, 49.72, 77.81, 155.42, 168.569. ESI-MS (positive ions, methanol): 241.2 [M+Na]+.

Synthesis of N'-Boc-Nα-Fmoc-aminoethylglycine: N_α-(N'-Boc-aminoethyl)glycine (0.3811 g) was dissolved in 10 ml of THF, then Fmoc-Cl (0.9 g) and DIEA (0.45 ml) were added. The reaction was stirred at room temperature for 1,5 h. After evaporating the solvent, ethyl acetate is added and the solution was washed with aqueos $KHSO_4$ sat., brine and dried over $MgSO_4$. The product was obtained as a white solid (yield: 25%). ^{1}H NMR ($CDCl_3$, 300MHz): δ(ppm) 1.30 (s, 9H, CH3 Boc), 2.94-3.08 (m, 2H, CH_2 aminoethyl), 3.10-3.36 (m, 2H, CH2 aminoethyl), 3.69-3.88 (m, 2H, CH_2 glycine), 4.12-4.24 (m, CH+CH_2 Fmoc), 7.20-7.30 (m, 6H CH aromatic Fmoc), 7.45-7.50 (m, 1H, CH aromatic Fmoc), 7.67-7.70 (m, 1H, CH aromatic Fmoc). 13C NMR ($CDCl_3$, 75MHz) δ(ppm): 28.29, 29.07, 31.66, 38.75, 46.75, 67.86, 78.93, 119.70, 125.07, 126.57, 127.03, 127.51, 127.85, 128.06, 128.54, 140.09, 143.91, 145.94, 148.33, 156.10, 157.98, 180.80. HRMS (ESI-MS, positive ions, methanol): calcd m/z for: $C_{24}H_{30}O_6N_2$ (MH+): 441.19421; found m/z: 441.20204

PNA synthesis: PNAs were synthesized either by manual submonomeric solid phase synthesis either automatically following protocols for standard PNA SPPS as already reported. PNAs were purified by semipreparative HPLC (final purity ≥90%) and characterized by HRMS ESI mass spectrometry, positive ions). PNA1 calcd m/z : 898.0248 ($MH3^{3+}$), 673.769 ($MH4^{4+}$), 539.215 ($MH5^{5+}$), 449.512 ($MH6^{6+}$) found m/z: 897.702, 673.527, 539.023, 449.354.
PNA2 HRMS ESI calcd m/z : 867.016 ($MH3^{3+}$), 650.512 ($MH3^{3+}$), 520.610 ($MH5^{5+}$) found m/z: 867.357, 650.769, 520.816.

UV Melting Curves: Hybrid solutions containing PNA (5 μM) and complementary DNA (5 μM) were prepared in a phosphate buffer (10 mM phosphate, 100 mM NaCl, pH = 7) and incubated at 90°C for 5 min. UV melting curves were recorded with a Lambda Bio Perkin–Elmer spectrophotometer by measuring the absorbance at 260 nm or 351 nm from 15° to 90°C. Melting temperatures were taken as the maximum of the first derivatives of the melting

curves. Van't Hoff plots were done according to the literature (Seitz O and Köhler O, 2001) by using a home-designed software.

Fluorescence measurements: Fluorescence measurements were performed by a Perkin Elmer Luminescence Spectrometer LS 55 equipped with a thermo-controlled water circulator. All solutions were prepared in fosphate buffer 10 mM phosphate, 100 mM NaCl, pH = 7). Concentrations (in strand) were 1 μM for each component. All samples were excited at 342 nm and the emission was monitored at 397 nm. Five scans were taken and averaged for each data point.

Fluorescence anisotropy measurements: Fluorescence anisotropy measurements were performed on the same solutions used for fluorescence analysis. They were excited at 342 nm and monitored at 397 nm. The integration time was 15s. Ten scans were taken and averaged for each data point.

5.3 RESULTS AND DISCUSSION

5.3.1 Design and synthesis of the PNA probe bearing a 2-carboxymethyl pyrenyl unit

The PNA1 probe bearing a 2-carboxymethyl pyrenyl unit has been designed by substituting a cytosine residue linked to the PNA backbone with a pyrene moiety, also connected to the backbone through a carboxymethyl linker (Figure 5.1). This new functionality can be easily obtained by using the commercially available 2-pyrenyl acetic acid. On the Norovirus model sequence : H-TCGCCCTCCC-NH_2, the middle base, (C), was substituted by the pyrenyl moiety, H-TCGCC(Pyr)TCCC-NH_2.

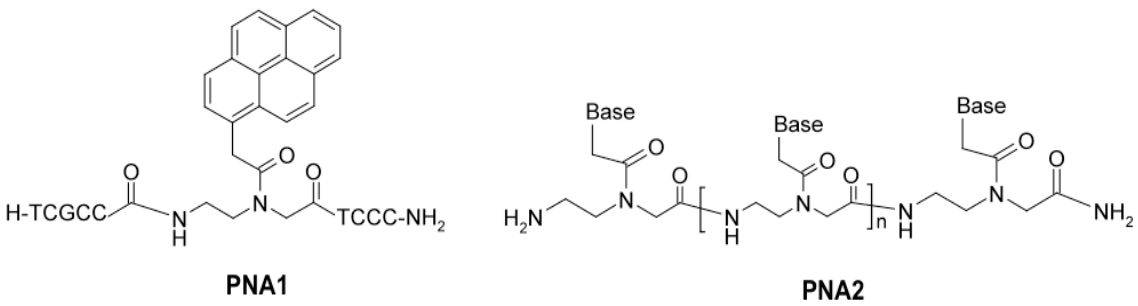


Figure 5.1. Chemical structure of a standard unmodified PNA (PNA2) and the pirenyl modified PNA (PNA1)

For comparison we synthesized also a conventional unmodified analogous PNA2 (Figure 5.1). The standard PNA and the modified PNA containing the 2-pyrenylacetyl moiety as nucleobase substitute were prepared by using the standard Boc solid phase synthesis protocol for the inclusion of the standard PNA monomers. The modified monomer was assembled directly on-resin by using the submonomeric approach (Sforza S et al., 2003).

First, the N'-Boc-N$^{\alpha}$-Fmoc-aminoethylglycine, the PNA backbone building block, synthesized according to literature procedures (Seitz O and Köhler O, 2001), was coupled to the solid support, followed by Fmoc deprotection directly on the resin.

The fluorophore pyrenyl acetic acid, opportunely activated (DIC/DhBTOH), was then inserted into the secondary amino group of the PNA aminoethylglycine backbone by amide bond formation. The PNA oligomer synthesis was then completed with the remaining monomers by standard procedures. With this approach we could obtain the desired PNA probe (PNA1, Table 1), which was subsequently purified by semipreparative HPLC, in fairly good yields.

The standard unmodified PNA2 was also synthesized for comparison. Both compounds showed a purity >90% by HPLC and were characterized by HRMS-ESI Mass Spectrometry.

5.3.2 Hybridization studies by UV measurements

In order to study the effect of the presence of the pyrenyl unit on PNA1 binding abilities, UV melting curves were recorded at 260 nm with the complementary oligonucleotides DNA1, DNA2, DNA3, DNA4, which differed only for the position of the nucleobase facing the pyrenyl unit (the sequences are reported in Table 5.1). The binding abilities towards a complementary RNA sequence (RNA1) were also tested.

The standard unmodified PNA2 was also tested against DNA1 and RNA1 for comparison (see Table 5.2).

The pyrenyl unit, consistently with the data previously reported (MacKinnon KF et al., 2007), was found to behave like an universal base: the melting temperatures against DNA1-4 were very similar in all cases, quite unaffected by the nucleobase directly facing the pyrenyl unit.

However, if compared to the unmodified PNA2, it is evident that the replacement of a nucleobase residue with the pyrenyl moiety induced a considerable loss in PNA-DNA duplex stabilities (Tm loss ranging between 10-12°C).

PNA-RNA complexes were found to be more stable than the corresponding PNA-DNA duplexes, consistently with the standard PNA behavior. Analogously to what observed for DNA, pyrenyl PNA1 formed a less stable PNA-RNA duplex than the unmodified PNA2.

UV melting curves were also recorded at 351 nm, the absorption maximum of the pyrenyl moiety. In all cases a decrease in the absorption was recorded upon heating. Therefore it is evident that, upon complexation of the PNA1 with the complementary nucleic acid (Figure 5.2), the pyrenyl unit undergoes a hyperchromic effect.

Table 5.1. PNA, DNA and RNA sequences used in this study. In bold the bases corresponding to the pyrenyl unit; in bold underlined the mismatched bases flanking the pyrenyl unit

Entry	Sequence
PNA1	H-TCGCC(**Aeg-CH2Pyr**)TCCC-NH2
PNA2	H-TCGCC**C**TCCC-NH2
DNA1	5'-GGGA**G**GGCGA-3'
DNA2	5'-GGGA**C**GGCGA-3'
DNA3	5'-GGGA**T**GGCGA-3'
DNA4	5'-GGGA**A**GGCGA-3'
DNA5	5'-GGGA**G**<u>**C**</u>GCGA-3'
DNA6	5'-GGGA**G**<u>**T**</u>GCGA-3'
DNA7	5'-GGGA**G**<u>**A**</u>GCGA-3'
DNA8	5'-GGG<u>**T**</u>**G**GGCGA-3'
DNA9	5'-GGG<u>**C**</u>**G**GGCGA-3'
DNA10	5'-GGG<u>**G**</u>**G**GGCGA-3'
RNA1	5'-rGrGrGrA**r**GrGrGrCrGrA-3'
RNA2	5'-rGrGrGrA**r**<u>**G**</u>**r**<u>**C**</u>rGrCrGrA-3'

Table 5.2. UV melting temperatures at 260 nm and 351 nm determined for PNA-DNA and PNA-RNA duplexes

Duplex	Tm 260 (°C)	Tm 351 (°C)[a]
PNA1 -DNA1	45.6	42.1
PNA1-DNA2	44.4	41.1
PNA1- DNA3	46.3	43.3
PNA1-DNA4	47.0	41.0
PNA1- RNA1	61.5	60.8
PNA2-DNA1	57.0	-
PNA2 -RNA1	70.4	-

Note: [a] at 351 nm a hyperchromic effect was observed upon binding, thus melting temperatures have been calculated as the minimum of the melting curves

The hyperchromic effect upon binding is opposite to what normally observed for the nucleobases and has already been reported for other pyrenyl probes (Wilson JN and Kool ET, 2006). Probably this behaviour can be due to a different orientation of the transition dipole of

pyrene compared with the standard nucleobases. This change in the chromicity effect indicates a change of the environment surrounding the pyrenyl unit going from duplex to single strand PNA.

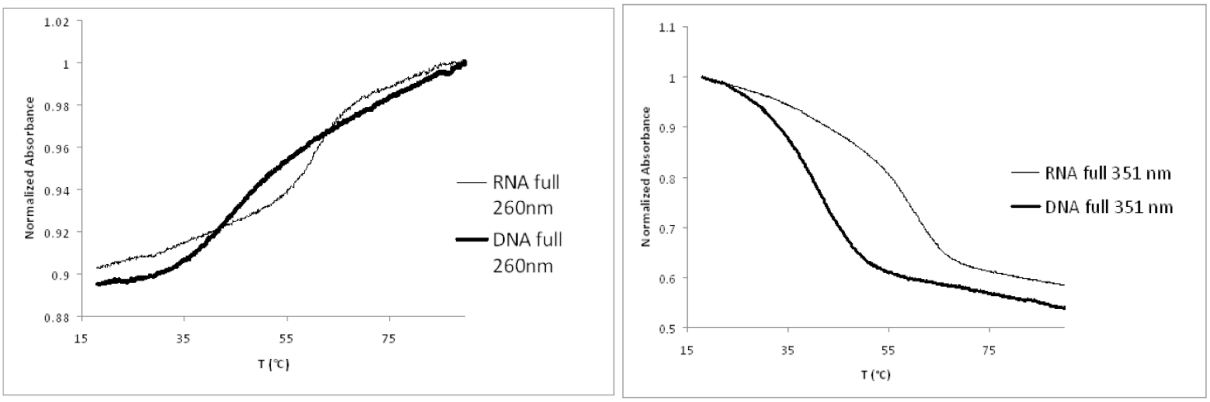


Figure 5.2. UV melting curves of PNA1/DNA1 complex (thickle lines) and PNA1/RNA1 complex (thin lines), at 260 nm (*left*) and at 351 nm (*right*). Measurement conditions are reported in the experimental sections

When calculating the melting temperatures from the curves recorded at 351 nm, a general decrease (3-6°C) was observed compared to those calculated at 260 nm for all the duplexes with the complementary DNA strands. Due to the lack of hydrogen bonding formation between the pyrenyl moiety and the nucleobases, the decomplexation of the fuorophorore slightly anticipates the actual melting of the duplex. In contrast, quite interestingly, only a slight difference of the melting temperatures calculated at the two different wavelenghts was observed for the PNA-RNA duplex, suggesting a stronger interaction with the proximal nucleobases of the pyrenyl moiety in RNA complexation, which is also consistent with the higher melting temperature observed. (Table 5.2).

5.3.3 Thermodynamic parameters of PNA-DNA and PNA-RNA duplex formation

In order to better understand the interactions occurring upon duplex formation in pyr-PNA-DNA and pyrPNA-RNA, UV melting curves (at 260 nm) were fitted by Van't Hoff plots, in order to derive enthalpic and entropic contributions. Thermodinamic data obtained for PNA1 were compared with those obtained for the analogue unmodified PNA2 (Table 5.3).

It is evident that the complex destabilization, as compared with the unmodified PNA2, can be mainly ascribed, in the case of the pyrPNA1-DNA1 duplex, to an enthalpic loss. This change can be interpreted as due to the steric hindrance induced by the pyrene moiety with the adjacent nucleobases, which hampers the hydrogen bond and the stacking interactions.

Table 5.3. Enthalpic and entropic contributions to PNA-DNA and PNA-RNA duplexes determined by Van't Hoff plots of the UV melting curves and free energies at 37°C

	ΔH° (kJ/mol) [(a)]	**ΔS° (J /°K mol)** [(a)]	**ΔG° (37°C kJ/mol)** [(a)]
PNA2-DNA1	-304	-819	-49.7
PNA1-DNA1	-237	-648	-35.5
PNA2-RNA1	-387	-1023	-69.5
PNA1-RNA1	-429	-1178	-63.1

Note: (a) Average relative standard deviation for the given values is 5%

The situation was found to be totally different for the pyr PNA1-RNA1 complex. The slight stability loss in this case was mainly caused by a more unfavourable entropic term, which came together with a consistent enthalpic gain. These data are consistent with a more favourable interaction between the pyrene unit and the RNA nucleobases. Thus, the thermodynamic data suggest that the pyrene favourably interacts with the nucleobases in the case of the PNA-RNA and not in the case of the PNA-DNA duplex. This behaviour may be related to the different preferential arrangement of the PNA-RNA (Brown SC et al., 1994) nucleobases (A type helix) as compared to the PNA-DNA (Menchise V et al., 2003) (B+A type helix) one.

5.3.4 Spettrofluorimetric analyses

The fluorescence properties of the free PNA1 probe and of the duplexes PNA- DNA and PNA-RNA were also studied. Fluorescence analyses were performed in phosphate buffer solutions: 10mM phosphate, 100 mM NaCl, pH=7 containing PNA1 alone or together with DNA or RNA strands at a 1μM concentration. Emission spectra were registered at 25 °C at λex= 342nm. The PNA1 fluorescent emission spectrum is characterized by two emission maxima: the former at 372 nm and the latter more intense at 397 nm. (Figure 5.3A). When the PNA probe was complexed to a complementary DNA strand, the fluorescence intensity descreased, indicating a quenching of the pyrene moiety. (Figure 5.3B)

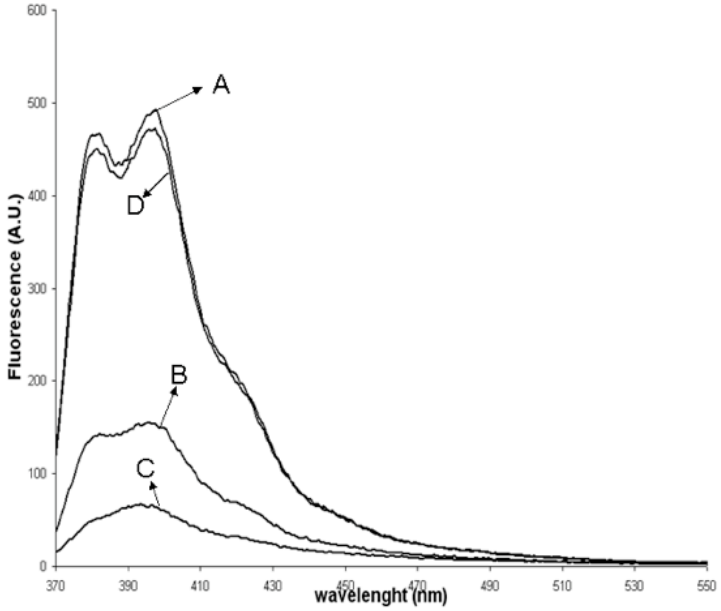


Figure 5.3. Fluorescence spectra of PNA1 alone (A), PNA1 complexed with DNA1 (B), PNA1 complexed with RNA1 (C) and PNA1 complexed with DNA5 (D). Measurements were carried out at 25°C for solutions containing each strand at a 1μM concentration in phosphate buffer.The excitation wavelegnht was 342nm

Albeit the effect was observed for all the complementary DNA1-4, it was much more evident for DNA1 and DNA2, probably due to the presence of the adjacent guanine and cytosine facing the pyrenyl unit, which are known to contribute to the quenching of the pyrene fluorescence emission, mainly via a photoinduced electron transfer from either neighbouring bases (Brown SC et al., 1994) (Figure 5.4).

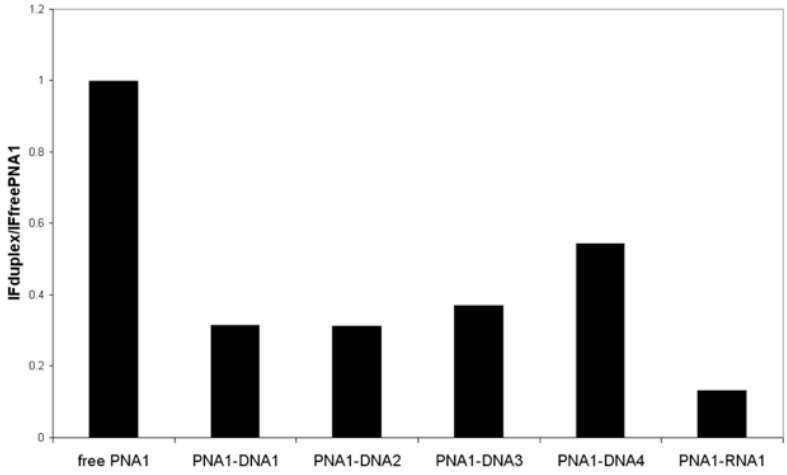


Figure 5.4. Fluorescence emission intensities at 397 nm normalized to the emission of free PNA1. Measurements were carried out as reported in Figure 5.3 and in the Experimental Section

The same behavior was also observed for the solutions containing PNA1 and RNA1 (Figure 5.3C), but in this case the fluorescence quenching was much more intense, indicating that the

pyrenyl unit interacts more tightly with the adiacent nucleobases, in agreement with the melting temperatures and the thermodynamic data discussed above.

In order to confirm that the specific fuorescence quenching observed was a consequence of duplex formation, a titration of PNA1 (1.2 μM) with increasing concentrations of DNA1 (from 0.2 to 2μM) was performed, by recording the fluorescence emission maxima at 397 nm. As it possible to observe in Figure 5.5, by plotting the fluorescence emission values at 397 nm against the [DNA1]/[PNA1] ratio, the signal decreased by increasing DNA concentration until the 1:1 ratio was reached, then it remained constant.

Since mixed sequence PNAs are known to form a 1:1 duplex with the complementary DNA, these data confirm that the observed specific fluorescence switch off is associated to the duplex formation.

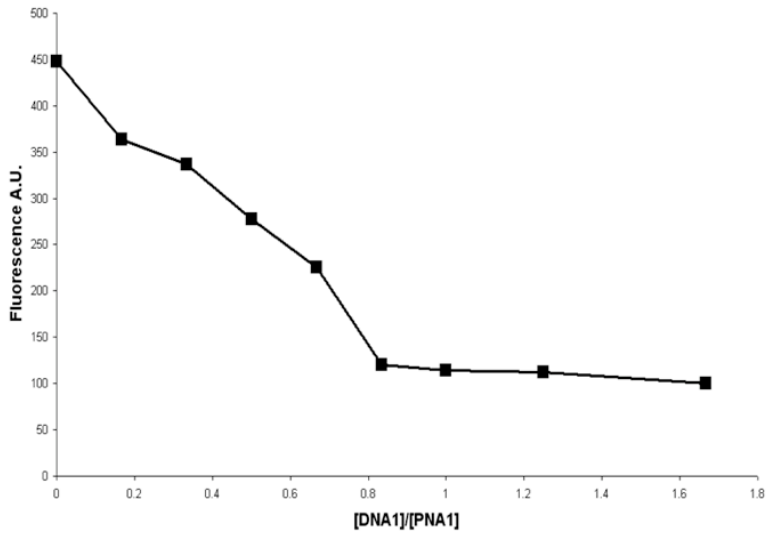


Figure 5.5. Titration curve performed by registering the fluorescence signal at 397 nm for solutions with PNA1 1.2 μM and increasing amount of DNA strand (from 0 to 2 μM). Measurments were carried out as reported in Figure 5.3 and in the Experimental Section.

5.3.5 Effect of mismatches in adiacent bases

With the aim of investigating the ability of the pyr-PNA to perform specific sequence recognition, the binding properties of PNA1 with DNA strands carrying mismatches in adjacent positions to the nucleobase G, which is opposite to the pyrenyl residue (Table 5.4), were studied.

All the possible mismatches were introduced either towards the 3' end (DNA5, DNA6, DNA7, carrying respectively C, T, A in place of G) and towards the 5' end (DNA8, DNA9,

DNA10, carrying respectively T, C, G in place of A). Also the mismatched RNA sequence RNA2 (C in place of G towards the 3' end) was considered.

Table 5.4. Melting temperature values (°C) for PNA-DNA and PNA-RNA duplexes calculated by the melting curves recorded at 260 nm. In the case of pyrenyl-PNA, melting curves have been recorded also at 351 nm

Duplex	Tm 260 (°C)	ΔTm [a] (°C)	Tm 351 (°C)	ΔTm [b] (°C)
PNA1-DNA5	45.5	0.1	n.d.	> -22
PNA1-DNA6	28.5	-17.1	26.7	-15.4
PNA1-DNA7	30.0	-15.6	n.d.	> -22
PNA1-DNA8	40.2	-5.4	36.5	-5.6
PNA1-DNA9	37.5	-8.1	31.8	-10.3
PNA1-DNA10	34.0	-11.6	30.4	-11.7
PNA2-DNA5	47.1	-9.9		
PNA2-DNA6	24.6	-32.4		
PNA2-DNA7	32.0	-25		
PNA2-DNA8	43.0	-14		
PNA2-DNA9	n.d.	> -37		
PNA2-DNA10	n.d	> -37		
PNA1-RNA2	46.0	-15.5	44.0	-16.8
PNA2-RNA2	48.0	-22.4		

Notes: (a) difference of fullmatch PNA1-DNA1 Tm value and mismatch Tm value at 260nm. (b) difference of fullmatch PNA1-DNA1 Tm value and mismatch Tm value at 351nm

The specific sequence recognition was firstly studied by determining the melting temperatures at 260 and at 351 nm. The melting at 260 nm were compared with the ones obtained for the unmodified PNA2.

At 260 nm, all the mismatched duplexes, except PNA1-DNA5 showed a stability decrease, as compared to the fullmatch duplex **PNA1-DNA1**, although to a less extent than that shown by unmodified PNA2. Indeed, the presence of the pyrenyl unit decreased the sequence specificity.

By considering the melting values obtained at 351 nm, the difference of Tm between the fullmatch and the mismatched duplexes were generally higher. In the case of the C-T mismatch (**PNA1-DNA8**) the smaller difference suggests that the pyrenyl group interaction was not particularly disturbed by the mismatch. In contrast, in the case of C-C and C-A mismatches (**PNA1-DNA5** and **PNA1-DNA7**) the differences were found to be much higher:

no transition involving the pyrenyl moiety was observed at 351 nm, suggesting that no interaction took place between the pyrenyl moiety and the DNA nucleobases in the duplexes.
The same PNA1 sequence was tested with the analougous **RNA2** with a C/G mismatch. In this case, comparable melting temperatures were observed both at 260 and 351 nm as well as a consistent decrease in duplex stability (-15.5 °C and -16.8 °C). It must be noted that RNA2 has the same sequence as DNA5, which showed no interaction between the pyrenyl and the nucleobases. In contrast, it appears that the pyrenyl unit is able to interact with the RNA nucleobases even in the presence of a mismatch.
The fluorescence properties of PNA1 hybridized to mismatched DNA and RNA strands was also studied, by recording the emission spectra at 25°C in the same conditions used for the fullmatch complexes.
As shown in Figure 5.3D and 5.6, PNA-DNA complexes containing single DNA mismatches failed to quench efficiently the pyrenyl moiety, and their emission spectra resulted always higher than those observed for the fullmatch duplex.

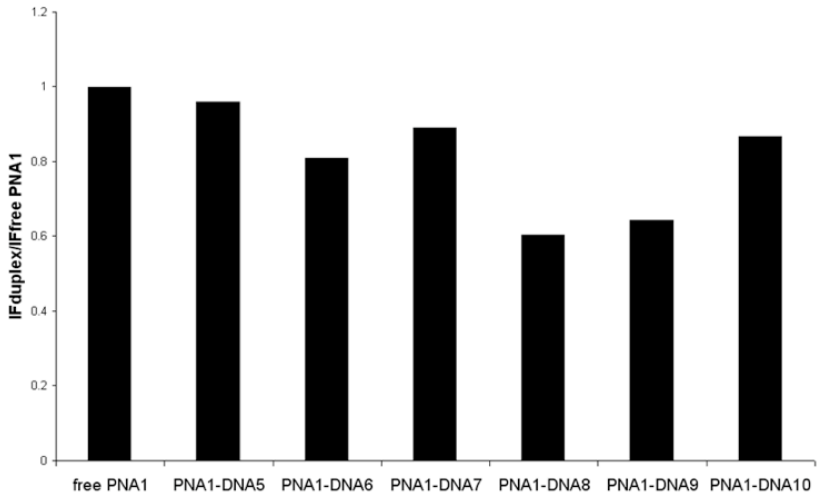


Figure 5.6. Fluorescence emission intensities at 397 nm normalized to the emission of free PNA1. Measurments were carried out as reported in Figure 5.5 and in the Experimental Section

Quite interestingly, the quenching was correlated with the occurrence of the transition measured at 351 nm for the mismatch duplexes. The duplexes not showing a Tm at 351 nm, (PNA1-DNA5 and PNA1-DNA7), were nearly not quenched, consistently with the absence of the pyrenyl interaction. Analogously, the mismatch complex with the high transition temperature (PNA1-DNA8) was the most quenched. All the other duplexes ranged between those two extremes.

A similar study was also performed by using the mismatched RNA2. In this case, the pyrenyl unit is expected to interact much more tightly with the RNA nucleobases. Accordingly, the fluorescence spectra at 25°C showed that the pyrenyl fluorescence was slightly switched off also in the case of the mismatch PNA1-RNA2 duplex.(Table 5.4).

However, the fluorescence spectra of the mismatched PNA1-RNA2 duplexes measured at 35 °C increased to levels comparable to that of free PNA1, thus increasing the recognition specificity (Figure 5.7)

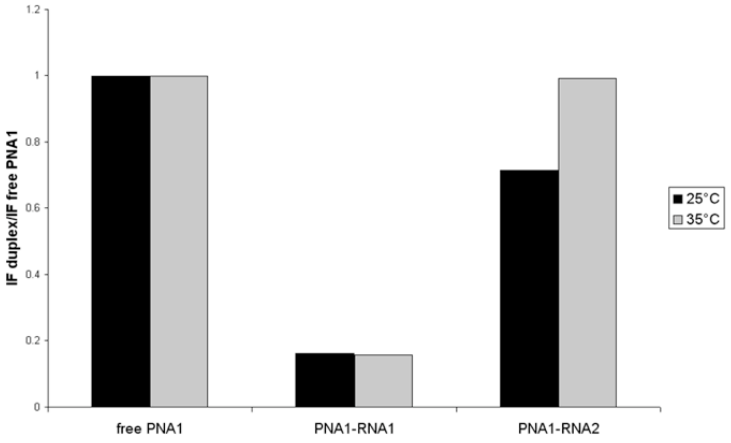


Figure 5.7. Fluorescence emission intensities at 397 nm at 25°C (black) and 35°C (grey), normalized to the emission of free PNA1. Measurments were carried out as reported in Figure 5.3 and in the Experimental Section

Finally, the mismatch PNA-DNA and PNA-RNA duplexes were also studied by fluorescence anysotropy, and compared to the full match complexes. The data reported in Table 5 showed that the anisotropy of the fluorescence emission of the pyrenyl group was very low in the case of free PNA1 and of the mismatch duplexes, (PNA1-DNA5 PNA1-RNA2), slightly higher than that measured for the isolated carboxymethylpyrene, indicating that both in the PNA alone and in the mismatch complexes the pyrenyl unit rapidly thumble in solution.

On the other side, a strong increase of the anisotropy values was observed for the fullmatch complexes, indicating a strong interaction of the pyrenyl unit upon duplex formation, which again was found to be higher in the case of the PNA-RNA duplex.

Table 5.5. Fluorescence Anisotropy (r) values calculated on the same solutions used for fluorescence analysis, excited at 342 nm and monitored at 397 nm

	Anisotropy (r)
2-Carboxymethyl-pyrene	0.010 ± 0.005
PNA1	0.035 ± 0.008
PNA1: DNA1	0.111 ± 0.015
PNA1:DNA5	0.032 ± 0.008
PNA1:RNA1	0.177 ± 0.022
PNA:RNA2	0.053 ± 0.007

5.4 CONCLUSIONS

The pyrenyl-PNA probe, here described, was found to form duplexes with complementary DNA and RNA strands with an enhanced RNA/DNA selectivity if compared with an analogous unmodified PNA sequence. The data suggest a stronger interaction with the proximal nucleobases of the pyrenyl moiety during RNA complexation. A specific fluorescence quenching was observed when the probe is complexed with the complementary strand of DNA and RNA, the effect being stronger in the case of RNA, confirming a tighter interaction.

A straighforward mismatch recognition was detected. Accordingly, the fluorescence is switched on in the presence of a mismatch if compared to a fullmatch duplex.

This work indicates that the PNA probe containing a pyrenyl unit in the place of a nucleobase enhances the ability of PNA to preferntially bind RNA than DNA. Thus, this type of probe can be a promising new tool for specific sequence recognition. Originally designed to target a norovirus sequence, the quenching effect upon hybridization makes the pyrenil-PNA not much attractive for real time assays for virus diagnostics. However, the probe showed a markedly different fluorescence behaviour in the presence of certain point mutations: since this system revealed high sensitivity for sequence variations even in a GC-rich environment, applications in the analysis of GC-rich sequences, such as the G-quadruplex (G4) forming regions (Burge S et al., 2006), might be envisaged. In particular, the peculiar fluorescence properties allow to visualize easily the presence of a fullmatch / single mismatch sequence especially in the case of a RNA target; this feature might be useful to detect rapidly mutating viruses or to be used to study the presence of G4-disruptive SNPs inside the guanine triplets involved in the G4 formation (Nakken S et al., 2009).

5.5 REFERENCES

Bichenkova EV, Gbaj A, Walsh L, Savage HE, Rogert C, Sardarian AR, Etchells LL, Douglas KT (2007). Detection of nucleic acids in situ: novel oligonucleotide analogues for target-assembled DNA-mounted exciplexes. Org Biomol Chem;5(7):1039-51.

Bichenkova EV, Sardarian AR, Wilton AN, Bonnet P, Bryce RA, Douglas KT (2006). Exciplex fluorescence emission from simple organic intramolecular constructs in non-polar and highly polar media as model systems for DNA-assembled exciplex detectors. Org Biomol Chem;4(2):367-78.

Bichenkova EV, Savage HE, Sardarian AR, Douglas KT (2005). Target-assembled tandem oligonucleotide systems based on exciplexes for detecting DNA mismatches and single nucleotide polymorphisms. Biochem Biophys Res Commun;332(4):956-64.

Brandt O, Hoheisel JD (2004). Peptide nucleic acids on microarrays and other biosensors. Trends Biotechnol;22(12):617-22.

Brown SC, Thomson SA, Veal JM, Davis DG (1994). NMR solution structure of a peptide nucleic acid complexed with RNA. Science;265(5173):777-80.

Burge S, Parkinson GN, Hazel P, Todd AK, Neidle S (2006). Quadruplex DNA: sequence, topology and structure. Nucleic Acids Res.;34(19):5402-15.

Christensen UB, Pedersen EB (2002). Intercalating nucleic acids containing insertions of 1-O-(1-pyrenylmethyl)glycerol: stabilisation of dsDNA and discrimination of DNA over RNA. Nucleic Acids Res;30(22):4918-25.

Conlon P, Yang CJ, Wu Y, Chen Y, Martinez K, Kim Y, Stevens N, Marti AA, Jockusch S, Turro NJ, Tan W (2008). Pyrene excimer signaling molecular beacons for probing nucleic acids. J Am Chem Soc;130(1):336-42.

Egholm M, Buchardt O, Christensen L, Behrens C, Freier SM, Driver DA, Berg RH, Kim SK, Norden B, Nielsen PE (1993). PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. Nature;365(6446):566-8.

Gbaj A, Walsh L, Rogert MC, Sardarian A, Bichenkova EV, Etchells LL, Whitcombe D, Douglas KT (2008). Target-assembled exciplexes based on Scorpion oligonucleotides. Biosci Rep;28(1):1-5.

Hrdlicka PJ, Babu BR, Sørensen MD, Harrit N, Wengel J (2005). Multilabeled pyrene-functionalized 2'-amino-LNA probes for nucleic acid detection in homogeneous fluorescence assays. J Am Chem Soc;127(38):13293-9.

Kumar TS, Madsen AS, Østergaard ME, Wengel J, Hrdlicka PJ (2008). Nucleic acid structural engineering using pyrene-functionalized 2'-amino-alpha-L-LNA monomers and abasic sites. J Org Chem;73(18):7060-6.

Leijon M, Mousavi-Jazi M, Kubista M (2006). LightUp probes in clinical diagnostics. Mol Aspects Med;27(2-3):160-75.

Lundin KE, Good L, Strömberg R, Gräslund A, Smith CI (2006). Biological activity and biotechnological aspects of peptide nucleic acid. Adv Genet.;56:1-51.

MacKinnon KF, Qualley DF, Woski SA (2007). Polycyclic aromatic hydrocarbons as universal bases in peptide nucleic acid. Tetrahedron Lett;48: 8074-8077.

Masuko M, Ohtani H, Ebata K, Shimadzu A (1998). Optimization of excimer-forming two-probe nucleic acid hybridization method with pyrene as a fluorophore. Nucleic Acids Res;26(23):5409-16.

Mayer E, Valis L, Wagner C, Rist M, Amann N, Wagenknecht HA (2004). 1-Ethynylpyrene as a tunable and versatile molecular beacon for DNA. Chembiochem;5(6):865-8.

Mayer-Enthart E, Wagenknecht HA (2006). Structure-sensitive and self-assembled helical pyrene array based on DNA architecture. Angew Chem Int Ed Engl;45(20):3372-5.

Menchise V, De Simone G, Tedeschi T, Corradini R, Sforza S, Marchelli R, Capasso D, Saviano M, Pedone C (2003). Insights into peptide nucleic acid (PNA) structural features: the crystal structure of a D-lysine-based chiral PNA-DNA duplex. Proc Natl Acad Sci;100(21):12021-6.

Nakken S, Rognes T, Hovig E (2009). The disruptive positions in human G-quadruplex motifs are less polymorphic and more conserved than their neutral counterparts. Nucleic Acids Res;37(17):5749-56.

Nielsen PE, Egholm M, Berg RH, Buchardt O (1991). Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science;254(5037):1497-500.

Okamoto A, Ochi Y, Saito I (2005). Fluorometric sensing of the salt-induced B-Z DNA transition by combination of two pyrene-labeled nucleobases. Chem Commun;(9):1128-30.

Paris PL, Langenhan JM, Kool ET (1998). Probing DNA sequences in solution with a monomer-excimer fluorescence color change. Nucleic Acids Res;26(16):3789-93.

Pellestor F, Paulasova P (2006). The use of peptide nucleic acids (PNAs) as probes in genetic and cytogenetic assays. Current Topics in Genetics, , 2006, 2 57-64.

Sakamoto T, Kobori A, Murakami A (2008). Microarray-based label-free detection of RNA using bispyrene-modified 2'-O-methyl oligoribonucleotide as capture and detection probe. Bioorg Med Chem Lett;18(8):2590-3.

Sahu N, Shilakari G, Nayak A, Kohli DV (2008). Peptide Nucleic Acids: A Novel Approach. Current Chemical Biology 2008, 2(2), 110-121.

Sforza S, Tedeschi T, Corradini R, Ciavardelli D, Dossena A, Marchelli R. Fast, Solid Phase Synthesis Of Chiral Peptide Nucleic Acids With A High Optical Purity By A Submonomeric Strategy.European Journal of Organic Chemistry, 2003; 1056-1063.

Seitz O, Köhler O (2001). Convergent strategies for the attachment of fluorescing reporter groups to peptide nucleic acids in solution and on solid phase. Chemistry;7(18):3911-25.

Seo YJ, Kim BH (2006). Probing the B-to-Z-DNA duplex transition using terminally stacking ethynyl pyrene-modified adenosine and uridine bases. Chem Commun;(2):150-2.

Socher E, Jarikote DV, Knoll A, Röglin L, Burmeister J, Seitz O (2008). FIT probes: peptide nucleic acid probes with a fluorescent base surrogate enable real-time DNA quantification and single nucleotide polymorphism discovery. Anal Biochem;375(2):318-30.

Tanaka K, Okamoto A (2008). Design of a pyrene-containing fluorescence probe for labeling of RNA poly(A) tracts. Bioorg Med Chem;16(1):400-4.

Totsingan F, Rossi S, Corradini R, Tedeschi T, Sforza S, Juris A, Scaravelli E, Marchelli R (2008). Label-free selective DNA detection with high mismatch recognition by PNA beacons and ion exchange HPLC. Org Biomol Chem;6(7):1232-7.

Wagner C, Rist M, Mayer-Enthart E, Wagenknecht HA (2005). 1-ethynylpyrene-modified guanine and cytosine as optical labels for DNA hybridization. Org Biomol Chem;3(11):2062-3.

Wang G, Bobkov GV, Mikhailov SN, Schepers G, Van Aerschot A, Rozenski J, Van der Auweraer M, Herdewijn P, De Feyter S (2009). Detection of RNA hybridization by pyrene-labeled probes. Chembiochem;10(7):1175-85.

Wanninger-Weiss C, Valis L, Wagenknecht HA (2008). Pyrene-modified guanosine as fluorescent probe for DNA modulated by charge transfer. Bioorg Med Chem;16(1):100-6.

Wilson JN, Kool ET (2006). Fluorescent DNA base replacements: Reporters and sensors for biological systems. Org Biomol Chem;4(23):4265-74.

Winnik FM (1993). Photophysics of preassociated pyrenes in aqueous polymer solutions and in other organized media**.** Chemical Review, 93(2), 587-614.

Yamana K, Ohshita Y, Fukunaga Y, Nakamura M, Maruyama A (2008). Bis-pyrene-labeled molecular beacon: a monomer-excimer switching probe for the detection of DNA base alteration. Bioorg Med Chem;16(1):78-83.

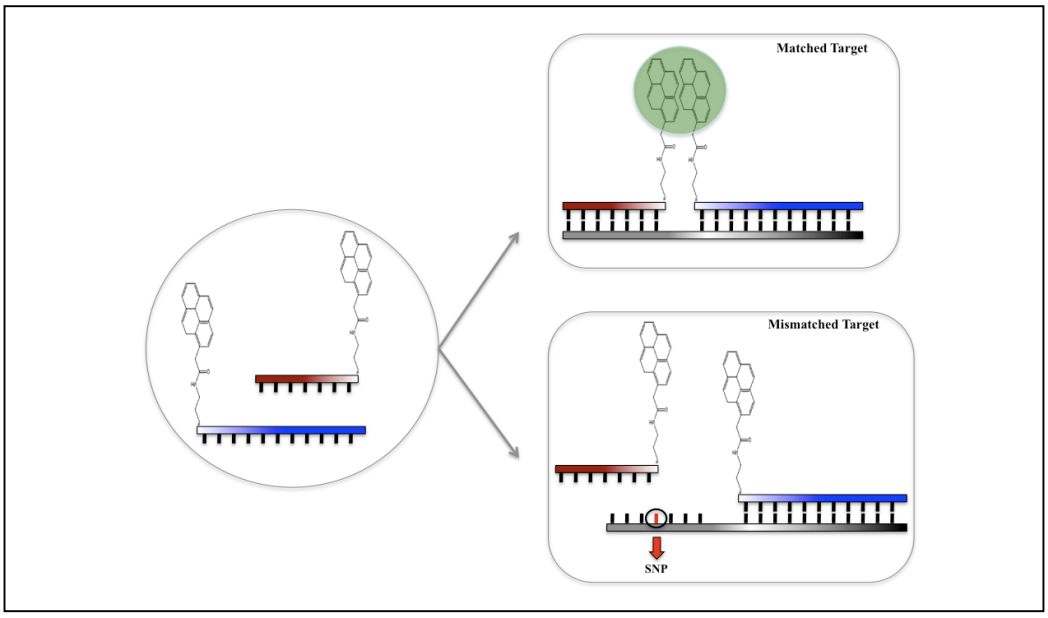
Chapter 6

Pyrene-conjugated PNAs as dual-probe system for PTPN22 C1858T polymorphisms detection

The problem of detecting single nucleotide polymorphisms (SNPs) is important in several areas, also in meedicine for the diagnosis of genetic-based deseases. Here we are taking in consideration the PTPN22 C1858T polymorphism sequence, implicated in several human autoimmune diseases including type 1 diabetes.

A PNA-based dual-probes system was designed, synthesized and charachterized. The two probes were derivatized with pyrene moieties in a proper orientation in order to provide excimer formation upon simultaneous hybridization. The dual-probes system was designed to be sequence specific with single-nucleotide resolution.

This kind of probes are of potential use for end-point analysis of single stranded DNA or RNA targets or for real time monitoring of amplification events in isothermal assays.



6.1 INTRODUCTION

Extensive knowledge on DNA sequences has been achieved in the last decade and consistent efforts are currently focused on the determination of the effects of individual variations in genomic sequences on cell functions. Single nucleotide polymorphisms (SNPs), has been revealed to be the most common type of mutation in human genome and are involved in single gene diseases such as haemophilia and cystic fibrosis, have a preeminent role in polygenic diseases such as diabetes and several types of cancers and are implicated in individual differences of immune system responses and in the aging processes. The basic research on SNP analysis is still ongoing and the development of effective methods to detect mismatches with single-base resolution is crucial for both research and diagnostic purposes. The current demand for robust, rapid and accurate methods for genotyping analysis, boosted the research of new synthetic chemical solutions for molecular biology and biotechnology applications.

Assays based on fluorescently labelled oligonucleotide probes are among the strategies most widely employed for sequence-specific analysis in solution (Marras SA, 2008). Well-established formats of fluorogenic probes rely on a combination of a fluorophore and a quencher molecule; these systems provide a detectable fluorescence change upon hybridization as consequence of probe transitions which can be conformational rearrangements as in the case of molecular beacons (Tyagi S and Kramer FR, 1996), Amplifluor (Nazarenko IA et al., 1997) and Scorpion primers (Whitcombe D et al., 1999), or enzyme-driven cleavages as in the case of TaqMan probes (Livak KJ et al., 1995). These strategies are particularly suited for homogeneous-based methods which are fast, less prone to cross-contamination events and do not require separation steps in order to remove the excess of the unbound probes. Although these probes are widely employed for reseach and routine genetic analyses, some limitations might arise attempting to target particular sequences or to detect small differences like SNPs: these kind of probes relies on differences in thermal stability between the fullmatched and the mismatched complexes and the assay conditions must be optimized in order to maximize these stability differences and fine-tune the single-base recognition capability. Sometimes, this operation might result cumbersome, expensive and time consuming and, for some SNPs, the assays migh result in low mismatch-detection resolouion.

Dual probes-based approaches represent an attrctive alternative of sequence-specific fuorescence-based detection methods. These systems require the simultaneous hybridization

of two oligomers on two contiguous sequences of a nucleic acid target. Both the probes involved in the dual system bear chemical groups able to interact to each other when placed at opportune distances and orientations and generate, *via* a transfer mechanism, detectable signals. Several dual probes-based systems have been developed exploiting florescence resonance energy transfer (FRET) (Cardullo RA et al., 1988; Masuko M et al., 2000), luminescence resonance energy transfer (LRET) (Sueda S et al., 2000), exciplex or excimer formation events. The latter two mechanisms are currently widely investigated in chemistry-based biotechnology and present some advantages over the other approaches. Usually based on the interaction between two polyaromatic fluorophores, the excimer-based systems require two identical molecules since the excimer phenomenon occurs when a molecule in the excited state interacts with another molecule of the same species in the ground state, whereas exciplex events occur upon formation of complexes involving two different molecules. In order to obtain efficient exicmer or exciplex formation, the orientation and the distance between the interacting aromatic molecules are crucial and must be accurately designed; these event are restricted to a parallel, cofacial configuration and the two moieties must have an interplanar distance of 3-4 Å (Masuko M et al., 1998), which is the order of thickness of a base pair. This result in a better theoretical resolution if compared with FRET systems, that usually operate over greater distances of 10-100 Å that corrispond to > ~3 bp.

Moreover, these systems may provide dramatic improvements in the background reduction, which can be extremely important when working under stringent conditions as in the SNP detection assays which make difficult the optimization steps for probes like molecular beacons. The excited state dimer formed during exciplex or excimer formation emits at longer wavelength providing a large Stokes-shift (usually more than 100 nm). Thus, each probe of the dual system give no signals at the detection wavelength and the signal emission depends on two hybridization events, which must occur simultaneously, reducing the risks due to aspecific signals and providing low background signals.

Among the polyaromatic molecules suitable for exciplex- and excimer- forming complexes, pyrene and pyrene derivatives currently play a pivotal role; the high quantum efficiency, the well-known photophysics and the flurescence behaviour, strongly dependent on the small environmental variations, make these molecules intresting fluorophore for probe labelling. Moreover, the relatively ease of pyrene-based derivatization chemistries have led to the development of several derivatization strategies based on the pyrene conjugation both at the backbone or at the nucleobases level. Pyrene molecules have been appended at various positions of the sugar units of locked nucleic acids (LNAs) (Hrdlicka PJ et al., 2005; Kumar

TS et al., 2008), of altritol nucleotides and ribonucleotides in hexitol nucleic acids (HNA) (Wang G et al., 2009) and phosphoroamidite derivatives in the place of a regular nucleobase (Paris PL et al., 1998). Moreover, direct conjugation at the backbone level has been reported for oligodeoxyribonucleotides (Christensen UB and Pedersen EB, 2002) and peptide nucleic acid (MacKinnon KF et al., 2007) and numerous works describing pyrene-conjugated nucleobases have also been reported, such as the Py-G (Wanninger-Weiß C et al., 2008), ^{Py}U , ^{Py}C and ^{Py}A (Okamoto A et al., 2005), PydU (Mayer-Enthart and Wagenknecht HA, 2006) and 1-Ethynylpyrene-modified guanine and cytosine (Wanger C et al., 2005). Moreover, other pyrene derivatives such as the ethynylpyrene or silylated pyrene molecules have beeen employed for probe derivatization.

Beside SNP detection and recognition, pyrene-derivatized probes have been designed for several purposes, such as labelling of RNA poly(A) tracts (Tanaka K and Okamoto A, 2008) or probing conformational DNA duplex transitions (Okamoto A et al., 2005; Seo YJ and Kim BH, 2006). Different types of probes such as linear probes, Scorpion oligonucleotides (Gbaj A et al., 2008) and molecular beacons (Conlon P et al., 2008; Yamana K et al., 2007) for homogeneus hybridization assays and linear probes for microarray-based label-free detection of RNA (Sakamoto T et al., 2008) have been investigated.

Pyrene-modified dual probes-based systems (Masuko M et al., 1998; Paris PL et al., 1998) have been employed showing good sensitivity for sequence-specific detection and SNP recognition. Pyrene-functionalized 2'-amino-LNAs in dual probes configuration have been used for optical mismatch discrimination exploiting both the superior properties of LNA for mismatch recognition and the sensitivity of pyrene molecules in the excimer state, for the local structural perturbation of ternary complexes (Umemoto T et al., 2007). Moreover, several exciplex forming probes have been investigated (Bichenkova EV et al., 2005; 2006; 2007) as a strategy to provide detectors that match specific needs or allow for multiplexing. Although pyrene binary probes coupled with time-resolved fluorescence spectroscopy have shown to be suitable for detecting mRNA in cellular extracts (Marti AA et al., 2006), the applications of these systems *in vivo* or for real time detection purposes is still far from being fully investigated.

Combining the oustanding properties of PNAs for mismatch recognition and the interesting characteristics of pyrene, we have created a PNA-based dual probes system (Figure 6.1) using the PTPN22 C1858T polymorphism as model sequence with the final aim to integrate this pyrene-PNA excimer-forming system in a real time isothermal-based assays for RNA detection or in an end-point detection method, targeting single stranded DNA or RNA.

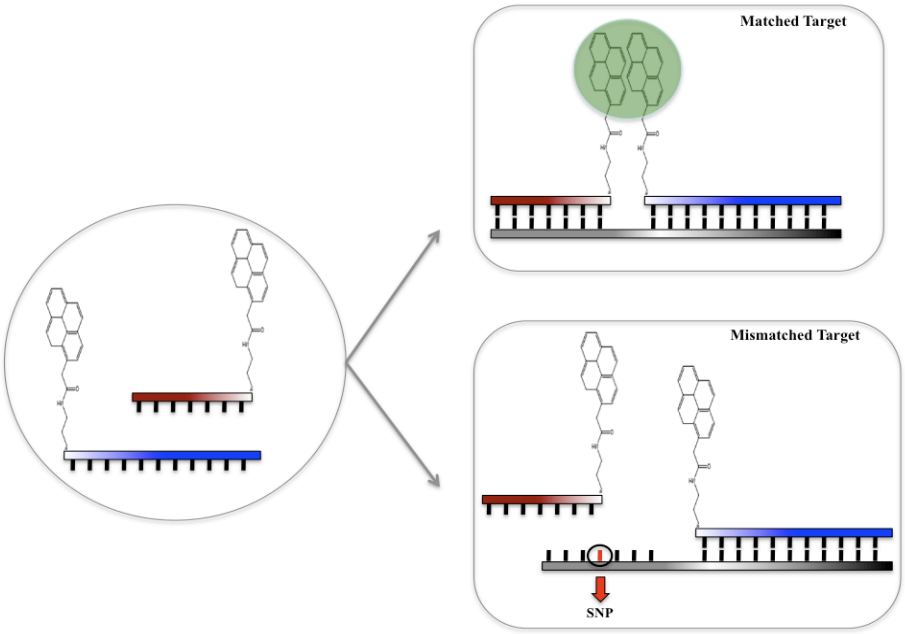


Figure 6.1. PNA based dual-probes based systems involving two pyrene-conjugated probes. Upon hybridization with the full matched target, the pyrene molecules are in the proper configuration to enable the formation of the excimer. The pyrenes are linked to the PNAs *via* side-chains of L-Lys.

The protein tyrosine phosphatase PTPN22 (also called LYP) seems to confer risk of developing diverse human autoimmune diseases, including type 1 diabetes, rheumatoid arthritis, autoimmune thyroid disease and systemic lupus (Gregersen PK et al., 2005); in particular, the Arg620Trp variant, which corresponds to the PTPN22 C1858T polymorphism, results in a gain of PTPN22 phosphatase activity in T cells (Gregersen PK et al., 2005). The role of PTPN22 in type 1 diabetes has been widely investigated in the last few years: this mutation site, localized in exon 14 of the PTPN22 gene, turned out to be one of the few non-HLA polymorphisms with relevant size effects in the inherited risk for type 1 diabetes (Zoledziewska M et al., 2008). Moreover, it has recently been elucidated that this polymorphism affects the development of T1D-associated autoimmunity only if children are exposed to cow's milk based formula during early infancy (Lempainen J et al., 2009); this supposed interplay between genetic and environmental factors remarks the needs for effective, rapid and inexpensive diagnostic methods for polymorphisms detection.

6.2 MATERIALS AND METHODS

Pyrenyl-PNA probes synthesis and characterization: PNAs were synthesized by using an ABI 433A authomatic synthesizer, using commercially avaible PNA monomers (Applied Biosystems) and following Fmoc synthesis protocols for standard PNA solid phase synthesis. Pyrenyl-lysine derivatized monomers were synthesized in the laboratories of the Departement of Organic and Industrial Chemistry (University of Parma) and inserted in the PNA oligomers by the standard PNA solid phase protocol.
PNAs were purified by semipreparative HPLC (final purity >80%) and characterized by ESI-MS.
Ex_PNA_Py, ESI-MS (H_2O positive ions): m/z: 791.8 $[MH_4]^{4+}$, 633.8 $[MH_5]^{5+}$, 528.4 $[MH_6]^{6+}$, 453.2 $[MH_7]^{7+}$
Py_PNA_SNP, ESI-MS (H_2O positive ions): m/z: 760.5 $[MH_3]^{3+}$, 570.6 $[MH_4]^{4+}$, 456.7 $[MH_5]^{5+}$. VEDI TU COME CHIAMARE LE DUE SONDE

Melting temperature measurements: Melting temperature (Tm) measurements were performed using a Perkin Elmer Lamda Bio 20 UV/Vis spectrometer and quartz cuvettes. All the experiments were carried out in buffered solutions (100 mM NaCl, 10 mM NaH_2PO_4 pH 7.0) at a 5 μM concentration and 1 ml as final volume. Where specified, the same buffered solution was employed adding 50% v/v of trifluoroethanol (TFE). Samples were heated up to 90 °C and cooled to room temperature. The melting curves were acquired between 18°C and 90°C with a temperature gradient of 1°C min^{-1} and the Tm of each sample was calculated from the maximum of the first derivative of the corresponding melting curves.

Fluorescence mesurements: i) Studies on the interaction of the probes with synthetic oligonucleotides were carried out in buffered solutions (100 mM NaCl, 10 mM NaH_2PO_4 pH 7.0) or in buffered solutions adding 25% or 50% of TFE v/v or 15% DMSO v/v using fluorescence quartz cuvettes and a final volume of 100 μl. If no different concentrations are specified, all the measurement were acquired at 0.5 μM, with a Perkin Elmer LS 55 spectrofluorometer using 347 nm as excitation wavelength, with $slit_{Ex}$ = 2.5 and $slit_{Em}$ = 5.0. Five scans were taken and averaged for each data point.

6.3 RESULTS AND DISCUSSION

6.3.1 Probe Design

The design of the PNA-based dual-probes system is based on the sequence of the PTPN22 C1858T polymorphism (rs2476601); this gene fragment provides an interesting model sequence because of its mixed nucleobase composition and represents an attractive target for potential applications. Moreover, this nonsynonymous SNP causes an aminoacid substitution *via* mRNA translation of a different codon sequence (Table 6.1), thus opening the possibility to target the sequence polymorphism at the RNA level instead of the corresponding genomic DNA.

The probe design was performed according to the following principles and constraints:

i) *Pyrene-conjugation strategy*: in order to allow the two pyrene molecules to adopt the proper configuration once linked to a PNA extremity and allowing the excimer formation to occur upon hybridization, the pyrene molecules were linked *via* the side-chain of a lysine residue at the N-term or at the C-term of the two probes. With the aim of maximize PNA binding interactions, L-lysine residues were incorporated in PNA modified monomers. Recently it has been showed that the presence of amino acid residues in PNA structure can induce in some case an improvement in binding affinity (Tedeschi et al., 2005). In particular the presence of stereochemical centers, introduced by the amino acid residues, promote a helical preference in the PNA oligomer and if the handeness is righthanded (as the DNA itself) the duplex formed is more stable. Among the different modifications introduced, the presence of a positive charged residue (lysine or arginine) at the C 5 position of the PNA backbone with L stereochemistry in this case, has shown improved binding specificity and selectivity. Since L-lys residue in this position point out from the duplex and do not interfere in watson and crick interaction, this functionality can be useful for the anchorage of molecules like fluorophores (Figure 6.2), this is the case, or to covalently link the PNA oligomer su solid surface for the development of chips of functionalized materials.

ii) *Sequence orientation and probes sequence*: since the PNA-based dual-probes system is conceived to be used as end-point detection method for asymmetric PCR products or for real time monitoring in isothermal-based detection strategies such as NASBA, the probes were designed to hybridize the cDNA sequence of the targeted gene: the products of NASBA usually result in amplified reverse-complementary copies of the initially targeted RNA.

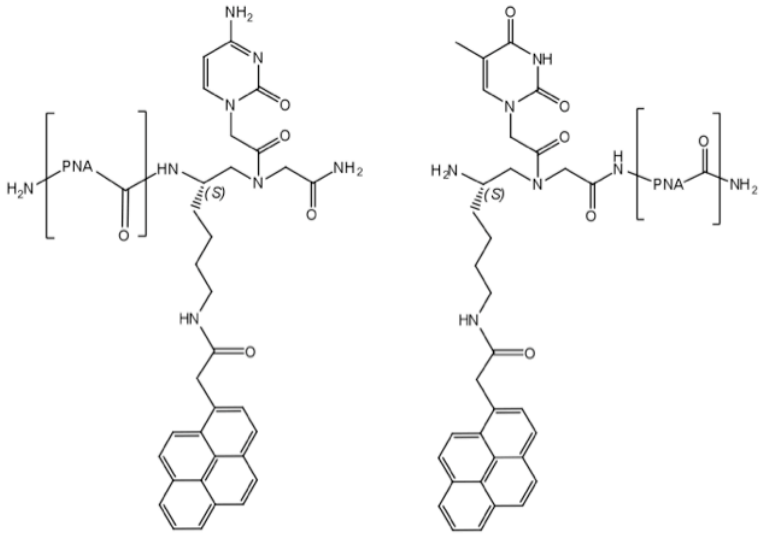


Figure 6.2. Structure of the Pyrenyl-lysine derivatized monomers used in the PNAs forming the dual probe system.

The PNA sequences were designed in order to minimize self-complementarity and probe-probe interactions. The SNP-discriminating probe was designed with a lower theoretical melting temperature (T_m) as compared with the secondary probe (ΔT_m of 10-15 °C): this configuration allows to easily employ the temperature as stringency parameter for the optimization of the SNP recognition and assures simultaneously a strong hybridization of the secondary probe which might be useful to open potential secondary structures.

Table 6.1. Information about the PTPN22 C1858T polymorphism (rs2476601) from the Reference SNP(refSNP) Cluster Report of the NCBI. The nucleotide sequence corrisponds to the mRNA strand; the mutation is reported in red and the sequences targeted with the PNA-based dual-probes system are underlined: Py_PNA_SNP (yellow), Ex_PNA_Py (grey).

Function	mRNA[(a)]			Protein		
	Accession	Position	Allele change	Accession	Position	Residue change
missense	NM_012411.2	1930	CGG ⇒ TGG	NP_036543.2	620	R [Arg] ⇒ W [Trp]
missense	NM_015967.3	1947	TGG ⇒ CGG	NP_057051.2	620	W [Trp] ⇒ R [Arg]

(a) 5'- GATGATGAAATCCCCCTCCACTTCCTGTAC/TGGACACCTGAATCATTTATTGTGGTTGAGG - 3'

6.3.2 Initial characterization of the PNA-based dual probes system: Tm studies, fluorescence and co-solvent effects

Since previous works reported the needs of co-solvents to promote the excimer formation upon probes hybridization (Bichenkova EV et al., 2005; Gbaj A et al., 2008), the first characterization experiments were focused on the study of the effects of the buffer composition on the excimer signal. The PNA-based dual-probes system was first tested using a common phospate buffer without any co-solvent and no excimer formation was observed at temperatures ranging between 17 °C and 30 °C (data not shown).

These results are in agreement with previous reports both for exciplex and excimer formation, in which the best results were achieved adding co-solvents such as TFE (Bichenkova EV et al., 2005; Gbaj A et al., 2008). In these approaches a 1-pyrenylmethylamine was attached to the oligonucleotides *via* the 3' terminal phosphate group. Also the dimethylformamide (DMF) was reported to increase the intensity of the excimer emission (Masuko M et al., 1998).

However, other dual-probes systems were reported to be able to form an excimer state without any co-solvent (Umemoto T et al., 2007); these different behaviours might be due to the different probe derivatization approach: Wengel and colleagues inserted the pyrene molecule at the N2' atom of 2'-amino-LNA monomers through a short and rigid carbonil linker, a configuration which seems to create sufficient structural constraints to allow the excimer state to occur upon probe hybridization.

We decided to conjugate the pyrene moiety *via* a lysine side-chain, which is a longer and less rigid; thus the excess of mobility might need to be compensated by tuning the reaction buffer in order to have the excimer formation. Thus, the PNA probes were studied in a phosphate buffered solution added with TFE or ethanol (25% or 50% v/v). All the conditions tested showed the formation of the excimer state upon probe hybridization with the fullmatch DNA oligonucleotide (Figure 6.3). However, the TFE 50% v/v (Figure 6-3b) and the ethanol 50% v/v (Figure 6.3d) seem to be more suited to provide high excimer signals upon the ternary complex formation.

Interestingly, the dual-probes system exhibited a good sensitivity in SNP recognition, resulting in a very low florescent emission in the presence of the mismatched DNA oligonucleotide corresponding to the wild type PTPN22 sequence, using all the buffer conditions tested.

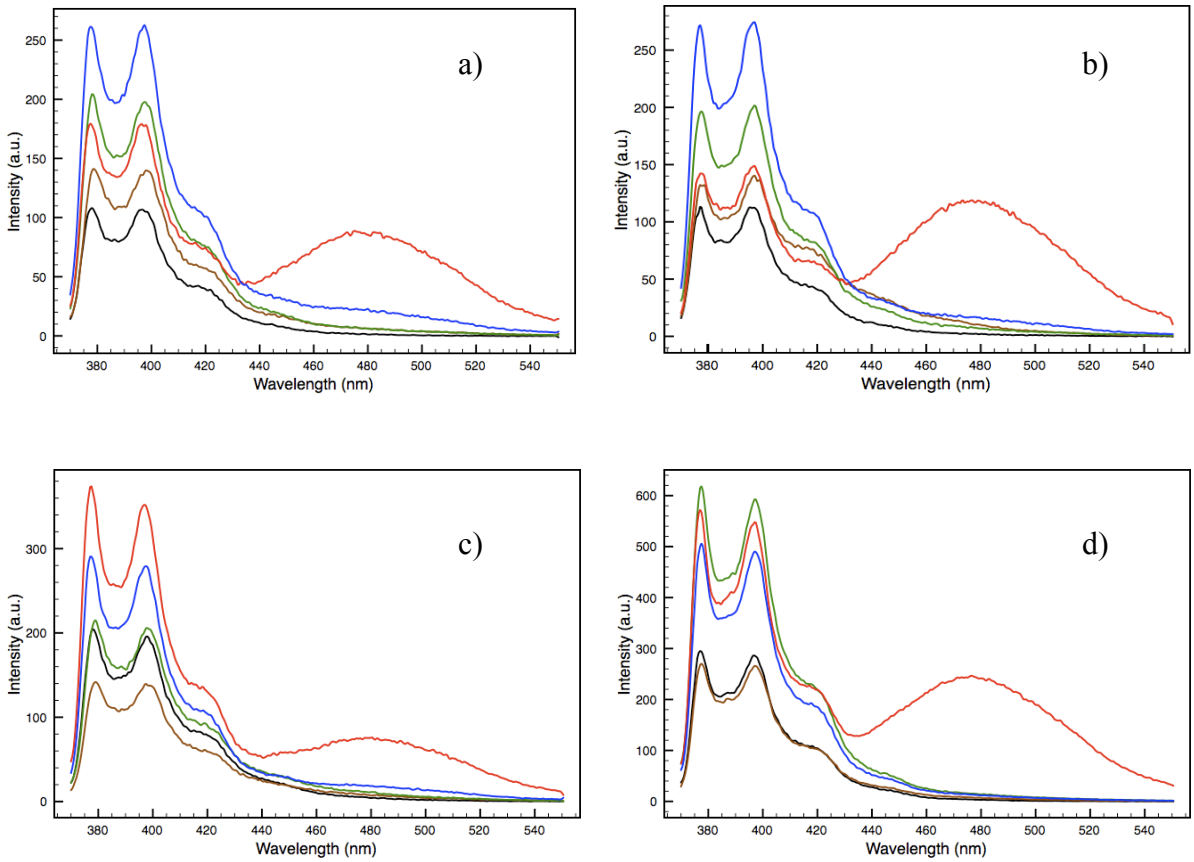


Figure 6.3. Fluorescence spectra of the Py_PNA_SNP (black), Ex_PNA_Py (brown), Py_PNA_SNP + Ex_PNA_Py (green), Py_PNA_SNP + Ex_PNA_Py + fullmatch target (red) and Py_PNA_SNP + Ex_PNA_Py + mismatch target (blue). Spectra were recorded at 25 °C; the concentration of each oligomer was 0.5 μM; the same experiment was repeated using a buffered solution (100 mM NaCl, 10 mM NaH_2PO_4 pH 7.0) with a) 25% TFE v/v; b) 50% TFE v/v; c) 25% EtOH v/v; d) 50 % EtOH v/v (excitation at 347 nm).

Moreover, differences in spectral change upon hybridization with the fullmatched DNA oligonucleotide were even evident at naked eye (Figure 6.4).

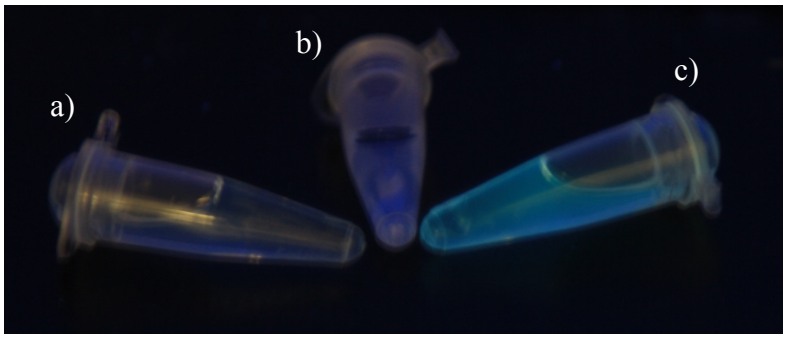


Figure 6.4. Photograph of solution containing the PNA-based dual-probes system (Py_PNA_SNP and Ex_PNA_Py) alone (a), in the presence of the wild type (b) and the mutated PTPN22 sequence (c). Each probe and target was used at 5.0 μM in buffered solution (100 mM NaCl, 10 mM NaH_2PO_4 pH 7.0) with 50% TFE v/v. The fluorescence was observed using an UV transilluminator at room temperature.

The same optimized buffer conditions ((100 mM NaCl, 10 mM NaH_2PO_4 pH 7.0, 50% TFE v/v) were used to analyze the T_m profile of the ternary complexes and the fluorescence emission as a function of the temperature (Figure 6.5). Moreover, the effect of TFE on the T_m of each PNA probe was evaluated individually and compared with the T_m obtained without the presence of co-solvent (Table 2).

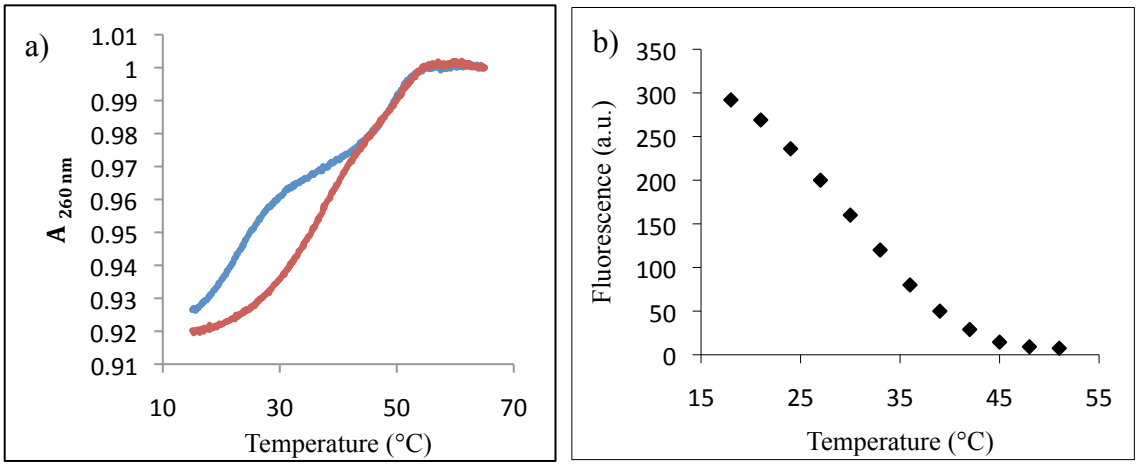


Figure 6.5. a) Melting curves (UV) of the ternary complexes containing the dual-probes system and the full match DNA oligonucleotide (red) or the mismatch oligonucleotide (blue). The measurements were carried out in buffered solution (100 mM NaCl, 10 mM NaH_2PO_4 pH 7.0) with 50% TFE v/v; the probes and the targets were used at 2.5 μM concentration and the temperature ramped at 1.0 °C/min. b) Fluorescence profile of the ternary complexes containing the dual-probes system and the full match DNA oligonucleotide as a function of the temperature. The measurements were carried out in buffered solution (100 mM NaCl, 10 mM NaH_2PO_4 pH 7.0) with 50% TFE v/v; the probes and the targets were used at 0.5 μM. Values correspond to the emission at 480nm.

Two different melting points, corresponding to the different PNA probe thermal stability, can be recognized in the melting profiles of the ternary complexes; this phenomenon resulted particularly pronounced when the dual-probes system was in the presence of the mismatch sequence because the thermal stability of the SNP-discriminating probe decreases (Figure 6.5a). On the other hand, the excimer fluorescence emission of the full matching ternary complex as a function of the temperature exhibited a profile with values that decrease at higher temperature in a way which mostly depend on the behaviour of the less stable probe, the Py_PNA_SNP; according to these results, the dual system in the used experimental conditions can be employed at a maximum temperature of approximately 35 °C, in order to have a satisfactory emission signal.

According to the melting temperature measured for the two probes, the presence of TFE in the reaction buffer gives rise to a destabilizing effect on both PNAs (Table 2); however this effect is stronger for the shorter probe (the Py_PNA_SNP).

Table 6.2. Melting temperatures of the single PNA probes forming the dual-probes system. The melting temperatures were measured using complementary DNA oligonucleotides with the same length of the probes. Values are reported in °C.

	Phosphate Buffer	Phosphate Buffer + TFE 50%
Ex_PNA_Py	51.7	49.0
Py_PNA_SNP	35.5	27.9

In order to better understand the capacity of the PNA-based dual-probes system to recognize the full match sequence over mismatches, the excimer signal was monitored in the presence of DNA oligonucleotides bearing single mismatches at different positions (Figure 6.6).

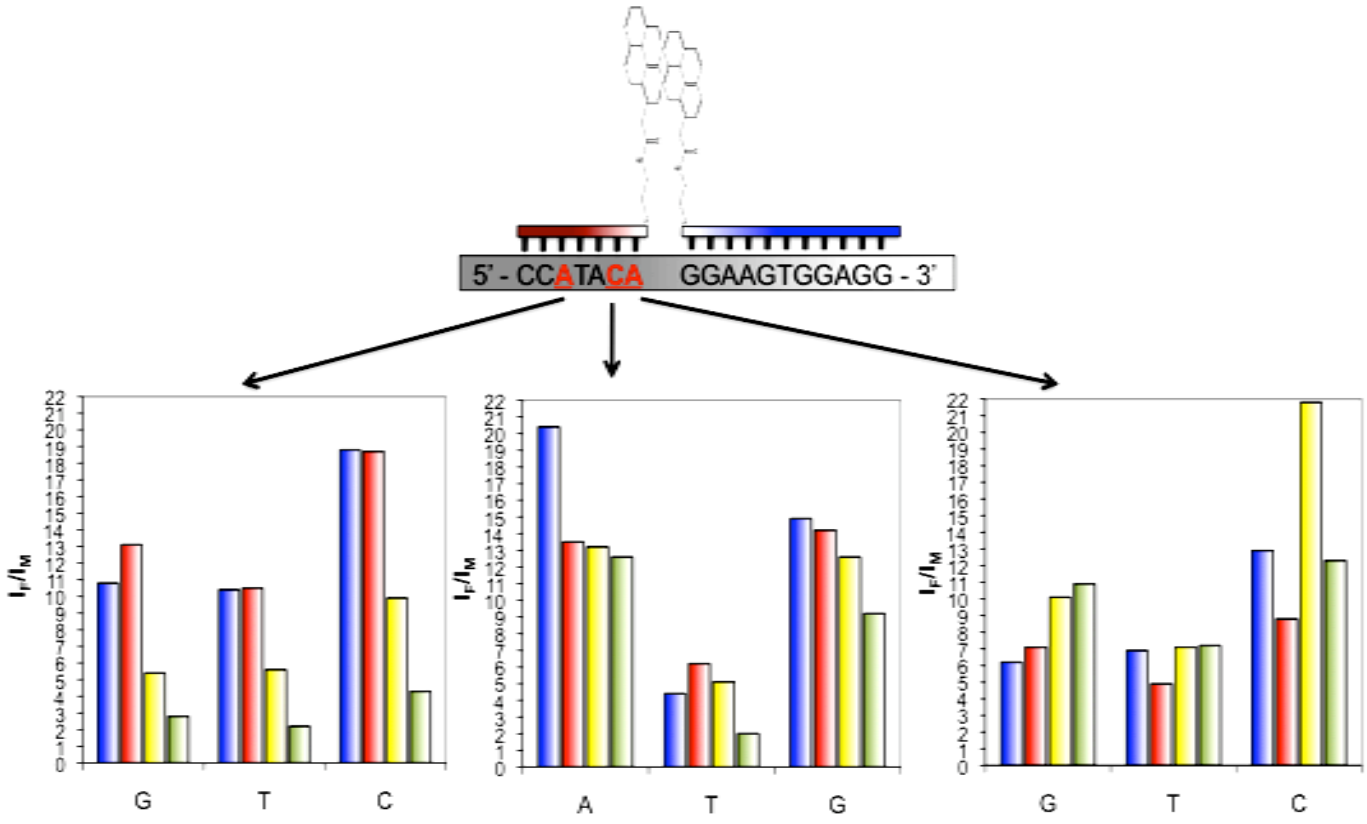


Figure 6.6. Relative intensity ($I_{\text{full match}} / I_{\text{mismatch}}$) of the excimer formation in the presence of oligonucleotides bearing single mismatches in three different positions (red nucleobases). The measurements were carried out at 25 °C in a buffered solution (100 mM NaCl, 10 mM NaH2PO4 pH 7.0) with 50% TFE (blue), 50% ethanol (red), 25% TFE (yellow), 25% ethanol (green) v/v as co-solvents; the probes and the targets were used at 0.5 μM. Values correspond to the emission at 480nm.

The dual system exhibited good performances in recognizing the fullmatch sequence; however, remarkable differences were observed depending on the type of nucleobase substitution, nucleobase position and buffer used. As a general trend, phosphate buffers containing TFE or ethanol at 50% v/v seem to be optimal conditions to increase the SNP recognition for the two internal mismatch positions; however, the co-solvents used at 25 % v/v give better or at least comparable results for the mismatch position closer to the pyrene molecules. Moreover, it is noteworthy that the relative fluorescence (I_F/I_M) for adenine substitutions in two different positions follows approximately the same trend (better recognition of cytosine than tymine and guanine) for all the buffers studied.

These results indicate the pivotal role of the solvent in the excimer formation and although our PNA-based dual-probes system exhibited good overall full match identification capabilities, the fine-tuning of the reaction contitions seems to be crucial in order to maximize sequence-specific detections. Moreover, the system showed good cababilities in detecting the mutations which correspond to the PTPN22 polymorphisms; this confirm the good probe design.

Since the dual-probe system was designed to target also RNA molecules, a first characterization using the full match RNA oligonucleotide was performed in order to compare the exciplex formation performances with the anologous DNA strand (Figure 6.7).

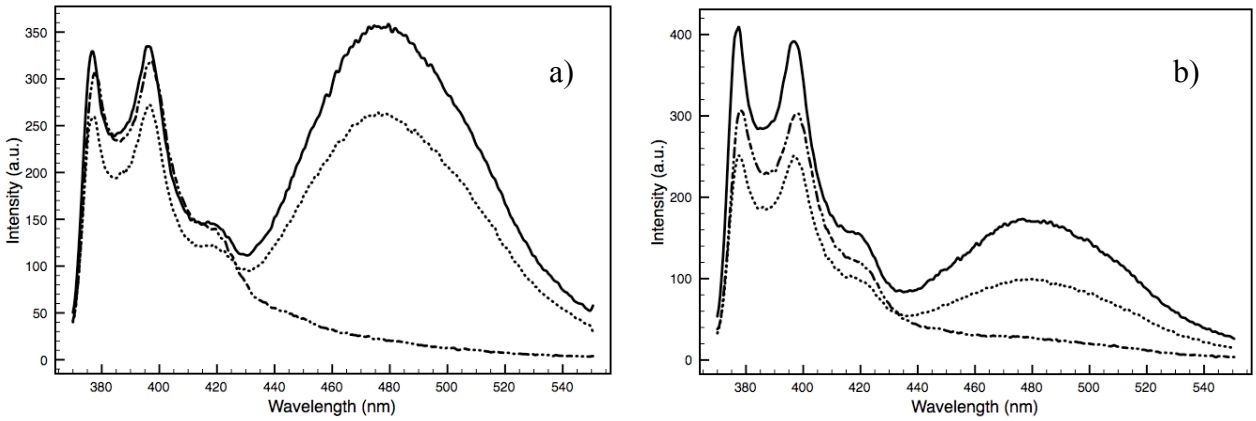


Figure 6.7. Fluorescence spectra of the dual complex alone (dash and dots), in the presence of the full match DNA (dots) or RNA (line) oligonucleotides. Spectra were recorded at 25 °C; the concentration of each oligomer was 0.5 μM; the same experiment was carried out using a buffered solution (100 mM NaCl, 10 mM NaH_2PO_4 pH 7.0) with a) with 50% TFE v/v or b) 25% TFE v/v (excitation at 347 nm).

Interestingly the dual-probes system exhibited a higher fluorescence signal corresponding to the excimer formation; since buffers containing high concentrations of ethanol or TFE impair the PCR-based reaction, we decided to test the probes using a buffer suited for real time

assays. We focused the attention on buffers containing 15-30% DMSO used in isothermal-based amplification strategies such as NASBA (Polstra AM et al., 2002). Preliminary results showed good performances using 15-20 % DMSO in phosphate buffer (data not shown) opening interesting applications of PNA-based excimer forming dual-probes system for fast screening and real time detection of SNPs located in coding mRNA regions or for studies of secondary and tertiary RNA structures. Moreover, since isothermal methods need shorter detection times and the RNA seems to be more UV resistant than DNA (Kundu LM et al., 2004), future applications of this kind of probes for detection of isotermally amplified RNA might be envisaged.

6.4 CONCLUSIONS

A PNA-based dual-probes system was designed, synthesized and characterized. The two probes were derivatized with pyrene molecules in the proper orientation in order to provide excimer formation upon simultaneous hybridization. The system was based on the PTPN22 C1858T polymorphism and the probes were designed to be sequence specific with single-nucleotide resolution. The emission signal occurred only upon ternary complex formation with the full match DNA sequence. However, in order to be effective, the system requires the presence of a co-solvent in the buffer, which resulted crucial for the fine-tuning of the mismatch recognition capability. These observations require further investigations in order to fully understand the molecular interactions using different solvents. Other linking strategies might be studied in order to introduce more rigid configurations and remove the need for co-solvents.

Moreover, the system worked well targeting RNA and seems to be effective in full match recognition in buffers containing DMSO; these kinds of buffers are commonly used in isothermal-based amplification assays such as NASBA.

These results reveal a potential use of this kind of probes for end-point analysis of single stranded DNA or RNA targets or for real time monitoring of amplification by isothermal methods.

6.5 REFERENCES

Bichenkova EV, Gbaj A, Walsh L, Savage HE, Rogert C, Sardarian AR, Etchells LL, Douglas KT (2007). Detection of nucleic acids in situ: novel oligonucleotide analogues for target-assembled DNA-mounted exciplexes. Org Biomol Chem;5(7):1039-51.

Bichenkova EV, Sardarian AR, Wilton AN, Bonnet P, Bryce RA, Douglas KT (2006). Exciplex fluorescence emission from simple organic intramolecular constructs in non-polar and highly polar media as model systems for DNA-assembled exciplex detectors. Org Biomol Chem;4(2):367-78.

Bichenkova EV, Savage HE, Sardarian AR, Douglas KT (2005). Target-assembled tandem oligonucleotide systems based on exciplexes for detecting DNA mismatches and single nucleotide polymorphisms. Biochem Biophys Res Commun. Jul 15;332(4):956-64.

Cardullo RA, Agrawal S, Flores C, Zamecnik PC, Wolf DE (1988). Detection of nucleic acid hybridization by nonradiative fluorescence resonance energy transfer. Proc Natl Acad Sci;85(23):8790-4.

Christensen UB, Pedersen EB (2002). Intercalating nucleic acids containing insertions of 1-O-(1-pyrenylmethyl)glycerol: stabilisation of dsDNA and discrimination of DNA over RNA. Nucleic Acids Res;30(22):4918-25.

Conlon P, Yang CJ, Wu Y, Chen Y, Martinez K, Kim Y, Stevens N, Marti AA, Jockusch S, Turro NJ, Tan W. Pyrene excimer signaling molecular beacons for probing nucleic acids. J Am Chem Soc. 2008 Jan 9;130(1):336-42.

Gbaj A, Walsh L, Rogert MC, Sardarian A, Bichenkova EV, Etchells LL, Whitcombe D, Douglas KT (2008). Target-assembled exciplexes based on Scorpion oligonucleotides. Biosci Rep;28(1):1-5.

Gregersen PK (2005). Gaining insight into PTPN22 and autoimmunity. Nat Genet;37(12):1300-2.

Hrdlicka PJ, Babu BR, Sørensen MD, Harrit N, Wengel J (2005). Multilabeled pyrene-functionalized 2'-amino-LNA probes for nucleic acid detection in homogeneous fluorescence assays. J Am Chem Soc;127(38):13293-9.

Kumar TS, Madsen AS, Østergaard ME, Wengel J, Hrdlicka PJ (2008). Nucleic acid structural engineering using pyrene-functionalized 2'-amino-alpha-L-LNA monomers and abasic sites. J Org Chem;73(18):7060-6. .

Kundu LM, Linne U, Marahiel M, Carell T (2004). RNA is more UV resistant than DNA: the formation of UV-induced DNA lesions is strongly sequence and conformation dependent. Chemistry;10(22):5697-705.

Lempainen J, Vaarala O, Mäkelä M, Veijola R, Simell O, Knip M, Hermann R, Ilonen J (2009). Interplay between PTPN22 C1858T polymorphism and cow's milk formula exposure in type 1 diabetes. J Autoimmun;33(2):155-64.

Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K (1995). Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl;4(6):357-62.

MacKinnon KF, Qualley DF, Woski SA (2007). Polycyclic aromatic hydrocarbons as universal bases in peptide nucleic acid. Tetrahedron Lett; 48: 8074-8077.

Marras SA (2008). Interactive fluorophore and quencher pairs for labeling fluorescent nucleic acid hybridization probes. Mol Biotechnol;38(3):247-55.

Martí AA, Li X, Jockusch S, Li Z, Raveendra B, Kalachikov S, Russo JJ, Morozova I, Puthanveettil SV, Ju J, Turro NJ (2006). Pyrene binary probes for unambiguous detection of mRNA using time-resolved fluorescence spectroscopy. Nucleic Acids Res;34(10):3161-8.

Masuko M, Ohuchi S, Sode K, Ohtani H, Shimadzu A (2000). Fluorescence resonance energy transfer from pyrene to perylene labels for nucleic acid hybridization assays under homogeneous solution conditions. Nucleic Acids Res;28(8):E34.

Masuko M, Ohtani H, Ebata K, Shimadzu A (1998). Optimization of excimer-forming two-probe nucleic acid hybridization method with pyrene as a fluorophore. Nucleic Acids Res;26(23):5409-16.

Mayer-Enthart E, Wagenknecht HA (2006). Structure-sensitive and self-assembled helical pyrene array based on DNA architecture. Angew Chem Int Ed;45(20):3372-5.

Nazarenko IA, Bhatnagar SK, Hohman RJ (1997). A closed tube format for amplification and detection of DNA based on energy transfer. Nucleic Acids Res;25(12):2516-21.

Okamoto A, Ochi Y, Saito I (2005). Fluorometric sensing of the salt-induced B-Z DNA transition by combination of two pyrene-labeled nucleobases. Chem Commun;(9):1128-30.

Okamoto A, Saito Y, Saito I (2005). Design of base-discriminating fluorescent nucleosides. J Photochem Photobiol C; 6 (2-3): 108-122.

Paris PL, Langenhan JM, Kool ET (1998). Probing DNA sequences in solution with a monomer-excimer fluorescence color change. Nucleic Acids Res;26(16):3789-93.

Polstra AM, Goudsmit J, Cornelissen M (2002). Development of real-time NASBA assays with molecular beacon detection to quantify mRNA coding for HHV-8 lytic and latent genes. BMC Infect Dis;2:18.

Sakamoto T, Kobori A, Murakami A (2008). Microarray-based label-free detection of RNA using bispyrene-modified 2'-O-methyl oligoribonucleotide as capture and detection probe. Bioorg Med Chem Lett;18(8):2590-3.

Seo YJ, Kim BH (2006). Probing the B-to-Z-DNA duplex transition using terminally stacking ethynyl pyrene-modified adenosine and uridine bases. Chem Commun;(2):150-2.

Sueda S, Yuan J, Matsumoto K (2000). Homogeneous DNA hybridization assay by using europium luminescence energy transfer. Bioconjug Chem;11(6):827-31.

Tanaka K, Okamoto A (2008). Design of a pyrene-containing fluorescence probe for labeling of RNA poly(A) tracts. Bioorg Med Chem;16(1):400-4.

Tedeschi T, Sforza S, Dossena A, Corradini R, Marchelli R (2005). Lysine-based peptide nucleic acids (PNAs) with strong chiral constraint: control of helix handedness and DNA binding by chirality. Chirality.;17 Suppl:S196-204.

Tyagi S, Kramer FR (1996). Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol. Mar;14(3):303-8.

Umemoto T, Hrdlicka PJ, Babu BR, Wengel J (2007). Sensitive SNP dual-probe assays based on pyrene-functionalized 2'-amino-LNA: lessons to be learned. Chembiochem;8(18):2240-8.

Wagner C, Rist M, Mayer-Enthart E, Wagenknecht HA (2005). 1-ethynylpyrene-modified guanine and cytosine as optical labels for DNA hybridization. Org Biomol Chem;3(11):2062-3.

Wang G, Bobkov GV, Mikhailov SN, Schepers G, Van Aerschot A, Rozenski J, Van der Auweraer M, Herdewijn P, De Feyter S (2009). Detection of RNA hybridization by pyrene-labeled probes. Chembiochem;10(7):1175-85.

Wanninger-Weiss C, Valis L, Wagenknecht HA (2008). Pyrene-modified guanosine as fluorescent probe for DNA modulated by charge transfer. Bioorg Med Chem;16(1):100-6.

Whitcombe D, Theaker J, Guy SP, Brown T, Little S (1999). Detection of PCR products using self-probing amplicons and fluorescence. Nat Biotechnol;17(8):804-7.

Yamana K, Ohshita Y, Fukunaga Y, Nakamura M, Maruyama A (2008). Bis-pyrene-labeled molecular beacon: a monomer-excimer switching probe for the detection of DNA base alteration. Bioorg Med Chem;16(1):78-83.

Zoledziewska M, Perra C, Orrù V, Moi L, Frongia P, Congia M, Bottini N, Cucca F (2008). Further evidence of a primary, causal association of the PTPN22 620W variant with type 1 diabetes. Diabetes;57(1):229-34.

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