Improved DNA hybridization parameters by Twisted Intercalating Nucleic Acid (TINA)

A model study on Hoogsteen based parallel triplex helix and Watson-Crick based antiparallel duplex helix formations

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THE THREE ORIGINAL PAPERS ARE

1. Schneider UV, Severinsen JK, Imrich G, Okkels LM, Jøhnk N, Mikkelsen ND, Klinge T, Pedersen EB, Westh H, Lisby G. A novel FRET pair for detection of parallel DNA triplexes by the LightCycler. BMC Biotechnology 2010, 10:4.

2. Schneider UV, Mikkelsen ND, Jøhnk N, Okkels LM, Westh H, Lisby G. Optimal design of parallel triplex forming oligonucleotides containing Twisted Intercalating Nucleic Acids – TINA. Nucleic Acids Research, 2010, Vol. 38, No. 13.

3. Schneider UV, ImrichG, Jøhnk N, Mikkelsen ND, Pedersen EB, Lisby G. Increasing the Analytical Sensitivity by Oligonucleotides Modified with *Para*- and *Ortho*-Twisted Intercalating Nucleic Acids – TINA. Plos ONE, June 2011, Volume 6, Issue 6, e20565.

INTRODUCTION

On a daily basis, medical doctors encounter patients with symptoms of infection. Based on the anamnesis, clinical examination, blood sampling and radiology, a number of working hypotheses are generated - some more likely than others. Often the most likely cause of disease is an infection of bacterial origin. Relevant clinical samples will be obtained followed by initiation of empiric antimicrobial chemotherapy. Preliminary results from the diagnostic microbiology laboratory will not be available for the first 24 hours, unless based on direct microscopy. The clinical outcome of a severe systemic infection is inversely correlated to the time of onset of appropriate antimicrobial chemotherapy (1). To cover most of the possible pathogens, the initial antimicrobial chemotherapy must contain one or more broad-spectrum antimicrobials. Positive microbial findings can subsequently be used to narrow the antimicrobial chemotherapy. Sometimes all culture-based results will be negative despite evident clinical infection and positive clinical effect of empiric antimicrobial chemotherapy. Obtaining new clinical samples will in most cases not clarify the clinical picture, since the microorganism(s) causing the disease has already been eradicated by the initial broad-spectrum antimicrobial chemotherapy.

Traditional clinical microbiology is based on growth of bacteria followed by species identification and antimicrobial susceptibility testing. The laboratory results are combined with guidance for interpreting results. The analytical sensitivity and impact on patient management of culture based diagnostics is limited by: i) obtaining a correct sample for microbiological testing, ii) contamination of samples from sterile body sites by the patient's own normal flora or the surrounding flora, iii) death of bacteria from time of sampling to inoculation in the microbiology laboratory, iv) choice of culture media and culture conditions, v) competitive growth of multiple bacteria with suppression of the causative microorganism by other microorganisms, vi) time for bacteria growth, and vii) risk of laboratory contamination (2;3).

Rapid detection of non-culturable microorganisms and slow growing microorganisms can be obtained by use of nucleic acid based target amplification systems e.g. the Polymerase Chain Reaction (PCR) (4-11). PCR assays are fast with an excellent analytical sensitivity and allow for detection of partly degraded microorganisms, but are also: i) dependent on efficient extraction of nucleic acids from the sample material, thus eliminating inhibitory factors, ii) relatively expensive, iii) require trained personnel, iv) prone to laboratory contamination, v) not optimal for quantification of microorganisms, and vi) necessitate being highly multiplexed to cover all relevant microbial targets (12;13). Signal amplification systems e.g. branched DNA (bDNA) allow for more accurate quantification of targets (14-16), but need improvements in analytical sensitivity, design simplicity and robustness (12;13).

In the future, target and signal amplification systems will need to improve the assay robustness to facilitate their use outside highly specialized laboratories. Moving the assays closer to the patient - using point-of-care diagnostics – allows the possibility of decreasing the total turn-around-time from sampling to action on the result (3). Other benefits of point-of-care diagnostics are increased speed for verification or rejection of working hypotheses helping to establish the correct clinical diagnosis and specific and targeted use of broad-spectrum antimicrobials. By

targeting use of broad-spectrum antimicrobials, the risk of secondary opportunistic infections and antimicrobial resistant bacteria can be minimized. Point-of-care diagnostics thereby improve the clinical impact of microbiological diagnostics on patient management (3).

A robust assay for point-of-care diagnostics must: i) be easy to handle, ii) be fast, iii) detect all microorganisms of interest in the sample – having a high analytical sensitivity, and iv) only detect the selected microorganisms – entailing no cross-reactivity to other targets. The analytical sensitivity and cross-reactivity of target and signal amplification systems can be manipulated by changing the stringency of assay conditions. By increasing the stringency, a decrease in cross-reactivity is obtained but at the cost of analytical sensitivity and *vice versa*. This problem can partly be solved by use of nucleic acid stabilizing molecules, which increase the analytical sensitivity without increasing the cross-reactivity of an assay (17-20).

This thesis describes the improved stabilizing effect on conventional Watson-Crick based antiparallel duplex helix formation and Hoogsteen based triplex helix formations by oligonucleotides modified with Twisted Intercalating Nucleic Acid (TINA). The stabilizing effect of TINA molecules in oligonucleotides is considered in relation to other nucleic acid stabilizing molecules. Finally, the use of TINA modified oligonucleotides and oligonucleotides modified with other nucleic acid stabilizing molecules for molecular diagnostics and therapy is discussed.

BACKGROUND

Nucleic acid structures

The basis of any nucleic acid structure is a sequence of nucleotides. Each natural nucleotide consists of a phosphate group, a furanose moiety - either 2'-deoxyribose (DNA) or a ribose (RNA) and a nitrogen-containing nucleobase (figure 1).

In DNA, the nucleotides are both containing purine nucleobases – adenine and guanine or pyrimidine nucleobases – cytosine and thymine. In RNA, the nucleobases adenine, guanine and cytosine are unchanged, whereas thymine is replaced by uracil. The nucleotides are linked together by covalent phosphodiester bonds that join the carbon-5' (c-5') of one furanose moiety to the c-3' of the next to form a nucleic acid strand (figure 1). Multiple nucleic acid strands assemble to form helixes with the nucleobases on the inside of the helix and the furanose moiety and phosphates on the outside of the helix. The thermal stability and conformation of any nucleic acid helix is regulated by: i) repulsion forces between the negatively charged phosphates internally in each nucleic acid strand and between multiple nucleic acid strands in the nucleic acid helix formation, ii) stacking interactions between the nucleobases in each nucleic acid strand and between the base pairs in the nucleic acid helix, and iii) hydrogen bond formations between pairs of nucleobases in the helix - contributing sequence specificity to the nucleic acids helix (21-23). Different types of nucleic acid helixes are described below:

The antiparallel duplex helix

The conventional antiparallel duplex helix is based upon base pairing between the thymine or uracil to adenine and guanine to cytosine nucleobases by respectively two and three hydrogen bonds (figure 2) (24). The two nucleic acid strands of the antiparallel duplex helix are in the 5' to 3' and 3' to 5' direction, thus in an antiparallel orientation (figure 3) (25).

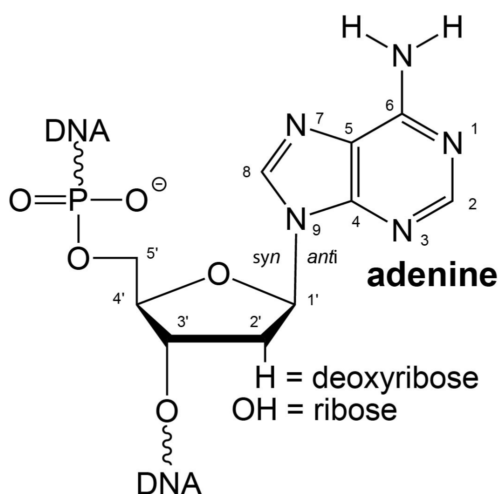

Figure 1
The structure of a dA or A nucleotide.

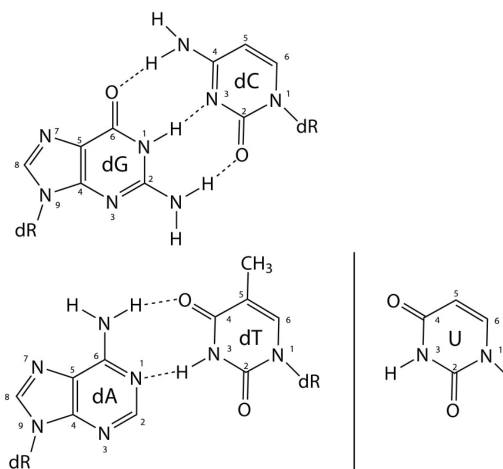

Figure 2
Base pairing between the guanine to cytosine nucleobases and the adenine to thymine or uracil nucleobases. dR deoxyribose and R is ribose.

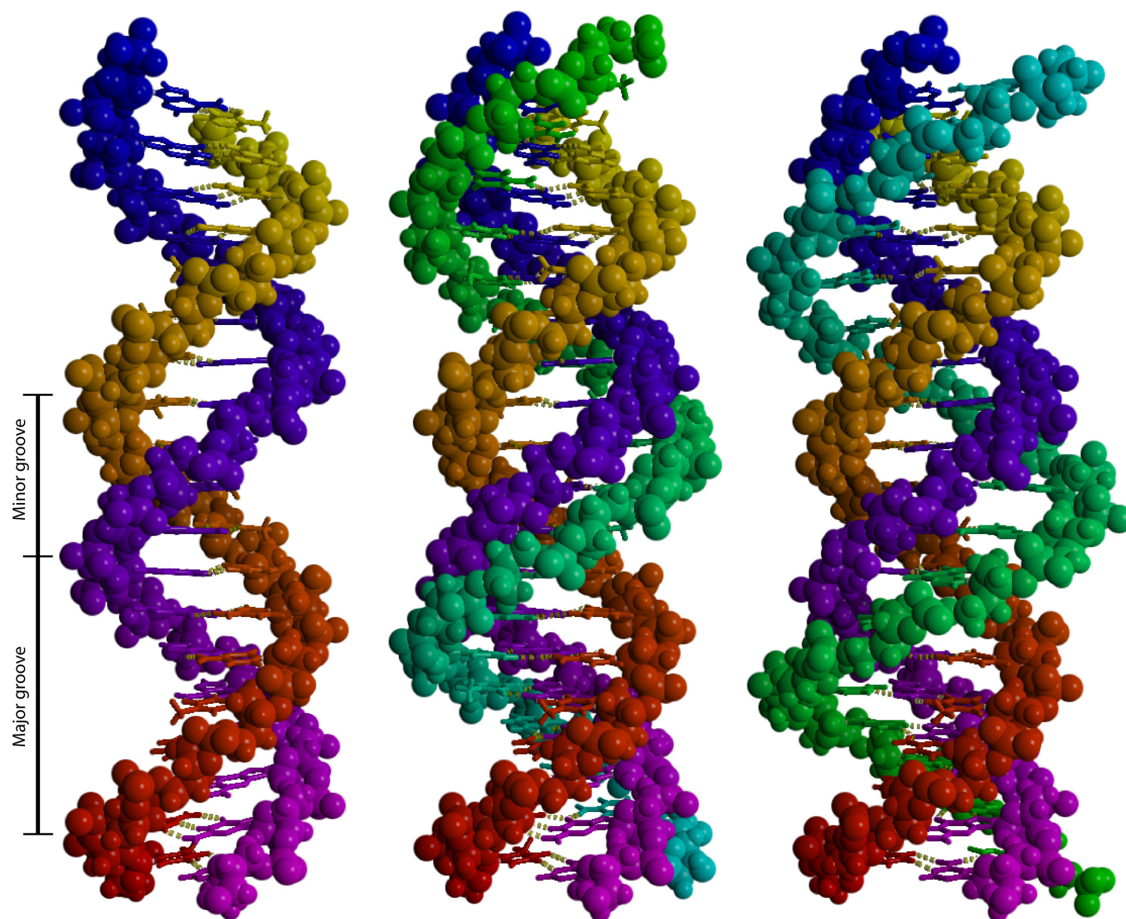

Figure 3
From left to right. A B-DNA antiparallel duplex a dC and dT nucleotide based parallel triplex and a dA and dG nucleotide based antiparallel triplex helix. Strand orientation 5′ → 3′: Blue → purple, red → yellow and green → cyan

Conformations of antiparallel duplex helixes

The conformation of DNA antiparallel duplex helix can be predicted by the hydration of the DNA and can be divided into three different helix conformations designated the A-DNA helix, the B-DNA helix and the Z-DNA helix conformation (26;27). Each of these differs at the nucleotide level regarding conformation of the 2′-deoxyribose and rotation of the nucleobase around the c-1′ (in the furanose moiety) to nitrogen-9 or n-1 (n-9 in purine and n-1 in pyrimidine nucleobases) glycosyl bond. In the 2′-deoxyribose the c-2′ and c-3′ can be twisted up from the plane of 2′-deoxyribose. When they are on the same side of the plane as c-5′ they are defined to be in the *endo* conformation (figure 4). Rotation of the nucleobase plane around the c-1′ to n-9 or n-1 glycosyl bond will either leave the 2′-deoxyribose and the nucleobase on each side of the glycosidic bond called the *anti* conformation or on the same side of the glycosidic bound called the *syn* conformation (figure 1). In A-DNA helix conformation the 2′-deoxyribose is in the c-3′ *endo* conformation and the nucleobase in the *anti* conformation, which makes the A-DNA helix conformation short and broad compared with the two other helix conformations (27). In B-DNA helix conformation the 2′-deoxyribose is in the c-2′ *endo* conformation and the nucleobase in the *anti* conformation (27). The B-DNA helix conformation is thin and long, whereas the Z-DNA helix conformation is even thinner and elongated compared with the A-DNA helix conformations (27;28). In Z-DNA helix conformation the pyrimidine nucleobase is in the *anti* conformation and the 2′-deoxyribose in the c-2′ *endo* conformation, whereas the purine nucleobase is in the *syn* conformation and the 2′ deoxyribose in c-2′ *exo* conformation (27;28). The conformation of RNA:RNA or RNA:DNA antiparallel duplex helixes have a A-DNA helix like conformation with the ribose of the RNA kept in the c-3′ *endo* conformation (29;30).

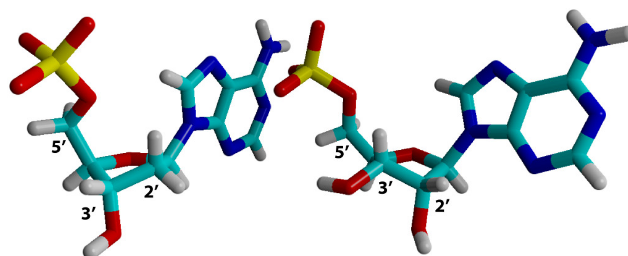

Figure 4
To the left a DNA nucleotide with the deoxyribose in c-2′ *endo* conformation. To the right a RNA nucleotide with the ribose in c-3′ *endo* conformation. Both nucleobases are adenine in the *anti* conformation

Major and minor groove in the B-DNA antiparallel duplex helix

The B-DNA helix has two grooves (figure 3). Since one is wider than the other, they are called the major and minor groove, respectively. In the B-DNA helix, the c-6, n-7 and c-8 of purines and c-4, c-5 and c-6 of pyrimidines are lined up in the major groove, whereas the c-2 and n-3 of purines and c-2 of pyrimidines are lining the minor groove (figure 2) (27;28).

The antiparallel duplex helix in the human genome

In the human genome the DNA antiparallel duplex helix is either in the B-DNA helix (predominately) or the Z-DNA helix conformation. Z-DNA helix conformations are favoured by nucleotide sequences with alternating pyrimidines and purines - especially d(CG:CG)$_n$ nucleotide sequences, which are frequently found in proximity to the transcription start site of genes (31). Z-DNA helix conformations are stabilized by methylation of c-5 in the cytosine nucleobase (figure 2) (28). The frequency of Z-DNA helix conformations has been linked to transcription activity, and Z-DNA helix conformations have been found to revert to B-DNA helix conformation if transcription is switched off (28;32). The stabilization of Z-DNA helix conformations by c-5 methylation of cytosine nucleobases is puzzling since c-5 methylation of cytosine in d(CpG) dinucleotides is well known to be associated with gene inactivation attained by transcriptional inhibition (33-35).

Hoogsteen based triplex helixes

Triplex helixes are formed intermolecularly between three nucleic acid strands (36) (figure 3) or intramolecularly between an antiparallel duplex DNA helix and a third strand provided by another area of the same antiparallel duplex DNA helix (named H-DNA) (37).

Conformation of triplex helixes

Triplex helixes are based on a homopurine nucleotide sequence in a nucleic acid target strand in 5' to 3' orientation. Only polypurine nucleotide sequences form stable triplex helixes, since each purine nucleobase simultaneously can form at least two hydrogen bonds with two other nucleobases (figure 5). The homopurine nucleotide sequence forms a Watson-Crick based antiparallel duplex helix with a complementary antiparallel polypyrimidine nucleotide sequence in 3' to 5' orientation (figure 3). The triplex forming oligonucleotide (TFO) sequence binds into the major groove of the DNA duplex in either parallel or antiparallel manner. Parallel triplex helixes (also named Hoogsteen triplex helixes) consist of a sequence of dC (2'-deoxyribosylcytosine-5'-monophosphate) and dT (2'-deoxyribosylthymine-5'-monophosphate) nucleotides in parallel orientation with the target polypurine nucleotide sequence (5' to 3') (figure 3) (38), whereas antiparallel triplex helixes (also named reverse Hoogsteen triplex helixes) consist of sequences of dG (2'-deoxyribosylguanine-5'-monophosphate) and dT or dG and dA (2'-deoxyribosyladenine-5'-monophosphate) nucleotides in antiparallel orientation with the target strand (3' to 5') (figure 3) (39). dC and dT nucleotide based parallel triplex helixes are pH dependent since they rely on protonation of the cytosine n-3 position (pKa equals 4.5) to form the two hydrogen bonds with the polypurine nucleotide target sequence (figure 5) (38;40;41).

Structural stability of triplex helixes

Parallel triplex helixes might be based on a RNA polypurine nucleotide sequence as the target strand, however, in order to form a parallel triplex helix with the maximum thermal stability, a DNA polypurine nucleotide sequence should be used as target strand (42-44). The two-polypyrimidine nucleotide sequences of the parallel triplex helix should preferably be two RNA polypyrimidine nucleotide sequences instead of two DNA polypyrimidine nucleotide sequences (42-44). RNA based TFO sequences can, in particular, stabilize the structure of both DNA and RNA based parallel triplex helixes in contrast to DNA based TFO sequences, which only have been found to stabilize DNA based parallel triplex helix formations (42-46). In antiparallel triplex helixes, all three nucleic acid strands in the triplex helix

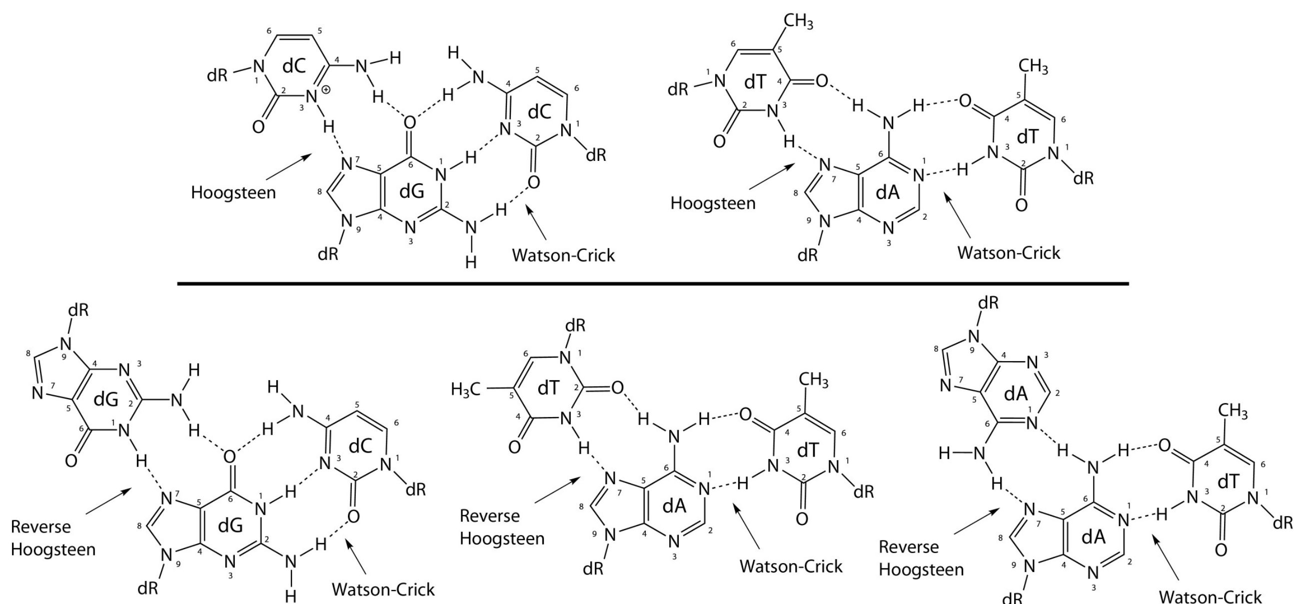

Figure 5
Nucleobase pairing in parallel triplex (top) and antiparallel triplex helixes (bottom)

should be DNA nucleotide sequences due to RNA based TFO sequences having not been found to form structural stable antiparallel triplex helixes (42). Parallel DNA triplex helixes are thermally stabilized by a higher percentage of C^{+}·GC deoxynucleotide triplets and alternating dC and dT nucleotides in the nucleotide sequence rather than longer sequences of the same nucleotide in the parallel triplex helix (47-50).

Impact of nucleobase mismatches on the thermal stability of triplex helixes

The ability of parallel triplex helixes to discriminate single nucleobase mismatches in the target nucleic acid strand is equal to, or higher than, the corresponding Watson-Crick based antiparallel duplex helix (51;52). Centrally placed nucleobase mismatches are more destabilizing compared to terminal nucleobase mismatches (53). Nucleobase mismatches have been found to increase the dissociation speed with no influence on the annealing of parallel triplex helixes (54). The formation of dG and dT nucleotide based antiparallel triplex helixes is faster than the formation of dA and dG nucleotide based antiparallel or dC and dT nucleotide based parallel triplex helixes (55;56). Despite faster annealing of dG and dT nucleotide based antiparallel triplex helixes the kinetic for formation of triplex helixes is significantly slower than the kinetics for formation of conventional Watson-Crick based antiparallel duplex helixes (57-61). The formation kinetic of triplex helixes can, however, be improved by adding triplex helix stabilizing molecules such as Neomycin (62-65).

Triplex helixes in the human genome

In the human genome, 1.9 million potential triplex helix forming nucleotide sequences with a minimum length of 15 purine nucleotides and a maximum of one pyrimidine nucleotide interruption have been identified (66;67). 97.8 % of all human genes have at least one potential triplex helix forming nucleotide sequence in the promoter or gene sequence and 86.5 % of all genes have a unique potential triplex helix forming nucleotide sequence (66). Likewise, potential triplex helix forming nucleotide sequences are over-represented in the coding strands of most bacteria genomes (68). Triplex helixes are important in gene regulation as they allow inhibition of gene transcription in promoter sequences and interfere with transcriptional elongation (reviewed in Wu, Q. *et al*) (66;69-72). The formation of triplex helixes in the human

genome have been associated with a number of diseases characterized by genetic instability (73), such as Friedreich ataxia (74) and autosomal dominant polycystic kidney disease (75). In Friedreich ataxia, 98 % of all patients have an expanded number of potential triplex helix forming $d(GAA:TTC)_n$ trinucleotide repeats in the first intron of the *fxn* gene (76). The expanded number of trinucleotide repeats is associated with the severity of the disease and inhibition of transcription of the *fxn* gene (74;77). These findings have lead to the hypothesis that the transcription inhibition is due to triplex helixes based on the $d(GAA:TTC)_n$ trinucleotide repeats (77)(reviewed in (74)). In autosomal dominant polycystic kidney disease, it has been suggested that genetic instability in a 2.5 kb polypurine:polypyrimidine nucleotide sequence in intron 21 of the *pkd-1* gene predisposes for the disease (78). H-DNA formations in the intron are believed to predispose to mutational deletions and insertions and, subsequently, loss of gene function (75).

Quadruplexes

Quadruplexes are predominately formed by hydrogen bond formation between four guanine nucleobases as G-quadruplex formations (figure 6) (79;80), however four cytosine nucleobases can also form a quadruplex formation named the i-motif formation at acidic pH (81).

Conformation of G-quadruplexes

All G-quadruplex formations rely on hydrogen bond formations between the four guanine nucleobases. The n-1, as well as the c-2 aminogroup, act as hydrogen donors whilst the oxygen of c-6 and n-7 act as hydrogen acceptors to form two hydrogen bonds with two other guanine nucleobases (figure 7). Quadruplexes consist of one, two or four nucleic acid strands in a variety of parallel and antiparallel combinations (82;83). The folding and unfolding of G-quadruplexes is very slow and may sometimes even suggest the possibility of triplex helix and parallel duplex helix intermediates (83-85). The thermal stability of RNA based parallel G-quadruplexes is increased in contrast to DNA based parallel G- quadruplexes in sodium buffers, whereas potassium buffers have been found to dramatically improve the stability of DNA based G-quadruplexes (84;86).

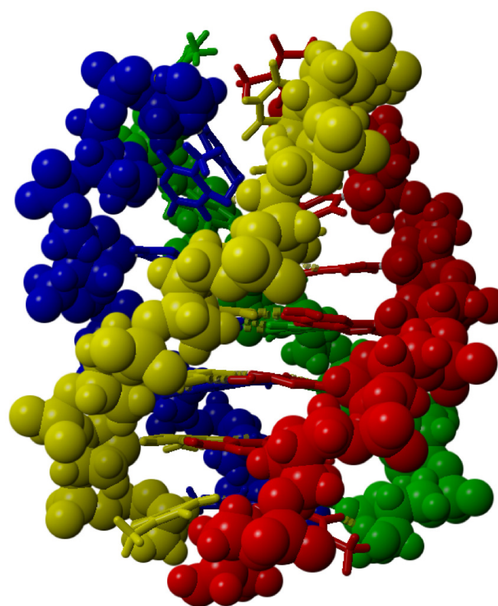

Figure 6
DNA G-quadruplex of the human telomeric $d(TTAGGG)_4$ with parallel strands. Based on NMR data from (79).

Formation of G-quadruplexes in the human genome

In the human genome, approximately 376.000 potential intramolecular G-quadruplex forming nucleotide sequences have been identified (87;88). G-quadruplex forming nucleotide sequences are significantly under-represented in coding regions of the genome (87;88). Potential intramolecular G-quadruplex forming nucleotide sequences have been identified in approximately 43 % of all gene promoters (89). Nucleotide sequences that allow the formation of G-quadruplexes are enriched 12-fold within the first 100 nucleotides up-stream from the transcription start site (89).

The formation of G-quadruplexes have been found to be involved in transcriptional regulation (90-95), meiosis (80;96), immunoglobulin heavy chain class switch recombination (80;97) and regulation of telomeres (98). Quadruplexes formed by the d(TTAGGG)$_n$ repeats of humane telomeres have been found to inhibit telomerase activity (99;100). In the cell, G-quadruplexes are resolved by RecQ helicases (101;102). Increased telomerase activity is found in approximately 85 % of cancers (103-106), whereas RecQ helicase deficiency is found in diseases characterized by premature ageing such as Werner syndrome and Bloom syndrome indicating that G-quadruplexes may play a crucial role in cellular homeostasis (107-109).

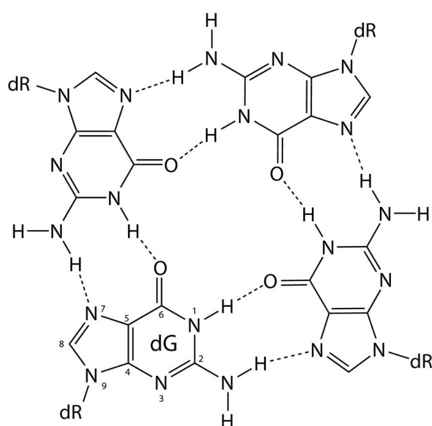

Figure 7
Nucleobase pairing in the G-quadruplex. dR is deoxyribose.

Hoogsteen based parallel duplex helixes

In the human genome, parallel duplex helixes might be formed as intermediates in formation of quadruplexes or as semistable duplex helixes at acidic pH (83;110-112). In the laboratory, parallel duplex helixes are used as controls in melting temperature (Tm) studies of parallel triplex helixes (113). Parallel duplex helixes are formed both intra- and intermolecularly but are distinctly less structurally stable than antiparallel duplex helixes, triplex helixes and quadruplexes (110;112;113). Conformationally, the parallel duplex helix equals a parallel triplex helix without the antiparallel duplex nucleotide strand. This leaves the homopurine nucleotide target strand in parallel orientation with a parallel nucleotide strand forming Hoogsteen based base pairs. Thus, the position of the parallel nucleotide strand on the targets strand equals the major groove of a homopurine nucleotide sequence of the target strand (figure 8) (112;114).

Nucleic acid stabilizing molecules

A variety of molecules have been developed to increase the structural and thermal stability of Watson-Crick based antiparallel duplex helixes and Hoogsteen based triplex helixes. (18;113;115-126). Some of these molecules are widely used, whereas others are known only by specialists within the scientific community. The molecules mentioned and described below are just some these molecules and it is beyond the scope of this thesis to account for the full diversity of nucleic acid stabilizing molecules in the scientific literature.

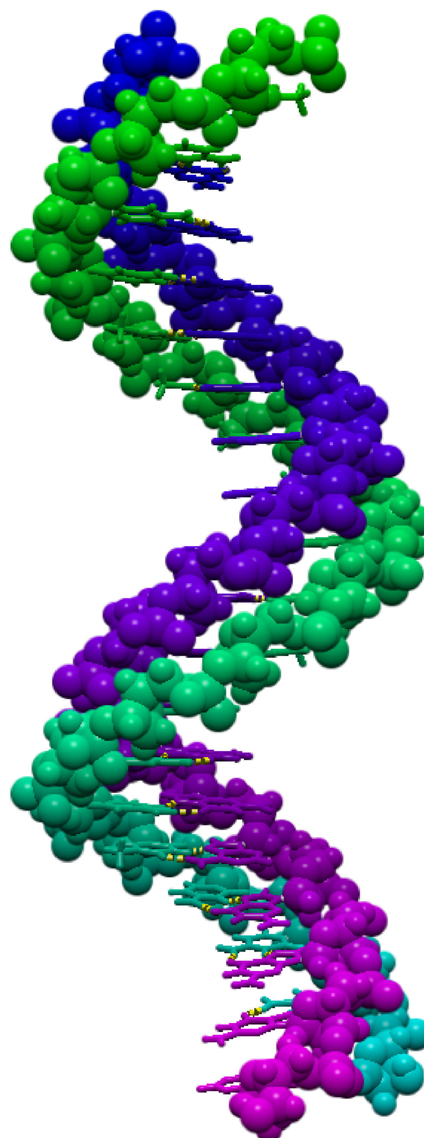

Figure 8
A parallel duplex helix.

Whilst not primarily developed to work as a fluorophore or quencher, the presented molecules are selected from among molecules that can be covalently inserted into the nucleotide sequence of shorter oligonucleotides. Some of the molecules that fulfill this restriction, although excluded from this presentation, are phosphorothioate DNA (PS), cyclohexene nucleic acid (CeNA), altritol nucleic acid (ANA), hexitol nucleic acid (HNA), 2’-*O*-methoxy-ethyl RNA (MOE), 2’-*O*-methyl RNA, Unlocked nucleic acid (UNA) and numbers of other molecules (119;127-136). The molecules described below have been selected as they are either commercially available, widely used or have properties that are especially relevant as compared to TINA molecules.

Ethylene-Bridged Nucleic Acid (ENA)

In the ENA molecule, the furanose moiety has been modified by a o-2' to c-4' ethylene bridge that locks the conformation of ENA containing oligonucleotides in an c-3' *endo* conformation (figure 9) (120;137). The binding affinity of ENA molecules to RNA equals the binding affinity of Locked Nucleic Acid (LNA) molecules (120), and ENA has been reported to be more nuclease resistant than LNA (120;138). Oligonucleotides with ENA are evident candidates as antisense oligonucleotides given that ENA are hydrophilic, nuclease-resistant and improve antiparallel duplex stability without compromising mismatch discrimination (120;137;139). Oligonucleotides containing ENA molecules have been found to increase the Tm of parallel triplex helixes by changing the conformation of the triplex helix to an A-DNA helix like parallel triplex helix (140). The TFO of a parallel triplex helix can be exclusively synthesized by ENA molecules and still allow the formation of a parallel triplex helix (140). ENA™ is a trademark of Mitsubishi-Kagaku Foods Corporation.

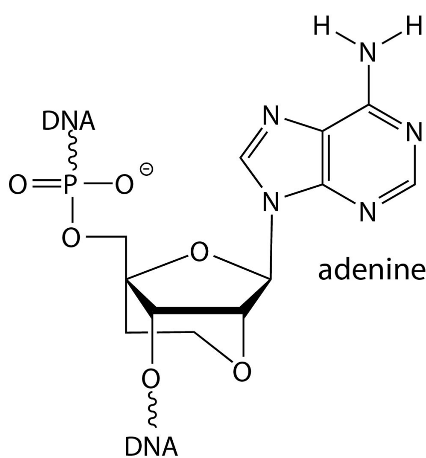

Figure 9
Structure of the adenine ENA nucleotide.

Intercalating Nucleic Acid (INA)

When inserted as a bulged insertion in the phosphate back-bone of nucleotide sequences or placed terminally on oligonucleotides (preferable), INA or (*S*)-1-*O*-(1-pyrenylmethyl)glycerol stabilizes DNA but not RNA (figure 10) (123). The term bulge insertion refers to the insertion of a molecule between two nucleotides in the phosphate back-bone of the oligonucleotide sequence. Two INA molecules should be placed with at least one nucleotide in between in a nucleotide sequence and the thermal stability increases as the two INA molecules are moved apart in the oligonucleotide sequence (123). When an INA molecule is placed directly next to a nucleotide mismatch, it reduces the destabilizing effect of the nucleotide mismatch on Tm of the antiparallel duplex helix (141). As the INA molecule is moved one or more nucleotides away from the mismatch in the antiparallel duplex helix, the mismatch discrimination is re-established (141). The thermal stability of an antiparallel duplex helix is reduced when two INA molecules are inserted into the two nucleotide sequences directly opposite each other (142). The combination of INA and LNA in oligonucleotides decreases the thermal stability of short DNA based antiparallel duplex helixes compared to oligonucleotides modified with LNA molecules only (143). INA molecules have been used i) to inhibit transcription start *in vitro* (144), ii) to reduce *kras* transcription in pancreatic cancer (Panc-1) cells through stabilization of a G-quadruplexes (145), and iii) to decrease background fluorescence in qPCR through stabilization of molecular beacons and thereby increasing the signal-to-noise ratio (146). The intellectual property rights of INA molecules are held by Human Genetic Signatures Pty Ltd (http://www.geneticsignatures.com/; March 14., 2011).

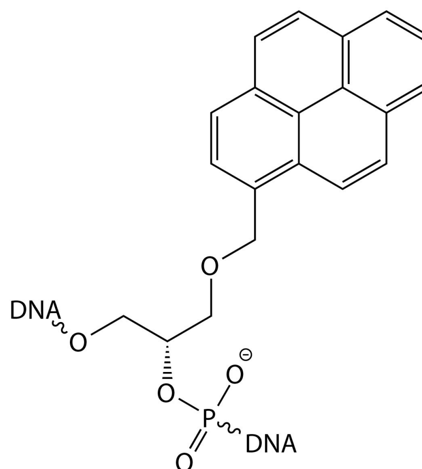

Figure 10
Structure of INA.

Locked Nucleic Acid (LNA)

In LNA, also known as Bridged Nucleic Acid (BNA), the furanose moiety has been modified by a o-2' to c-4' methylene bridge locking the pentose in the c-3' *endo* conformation (figure 11) (147;148). The c-3' *endo* conformation leads to an excellent increase in Tm stability and mismatch discrimination in antiparallel duplex helixes entailing LNA containing oligonucleotides (18;149). LNA containing oligonucleotides improve the analytical sensitivity and diminish the cross-reactivity of hybridization probes and are used in antisense strategies, particularly, due to their ability to bind strongly to RNA structures, excellent solubility in hydrophilic solutions and increased nuclease resistance of LNA containing oligonucleotides (150-152). In clinical diagnostics, LNA containing oligonucleotides have been introduced as hybridization probes in microarrays (153) and as PCR probes (154-158). LNA modified PCR probes and 3'-Minor Groove Binder (3'-MGB) modified probes have similar analytical sensitivity (154-158). In PCR primers, LNA containing oligonucleotides have been used to block abundant wild-type targets, allowing amplification of rare targets by conventional PCR primers (159-165). The design of LNA containing oligonucleotides depends on the target sequence, identity and position of mismatches, probe length and number as well as position of LNA nucleotides in the oligonucleotide sequence (166-169). In parallel triplex helixes, a central LNA nucleotide in the TFO sequence has been found to improve the Tm of the parallel triplex helix whilst maintaining a decrease in Tm caused by nucleobase mismatch (ΔTm) (170). Alternating LNA and DNA nucleotides in the TFO sequence increases the thermal stability of the parallel triplex helix, whereas a TFO sequence fully modified with LNA nucleotides shows no binding to the target DNA oligonucleotide sequence (171;172). The intellectual property rights for use of LNA molecules are held by Exiqon A/S with the exception of therapeutic applications rights, which are held by Santaris Pharma A/S. Exiqon A/S primarily develop assays for detection of microRNA using LNA containing oligonucleotides by

microarray, reverse transcriptase PCR (RT-PCR), northern blot and in situ hybridization (http://www.exiqon.com/; March 14., 2011). Santaris Pharma A/S is developing a range of different therapeutics for targeting intracellular RNA structures with LNA containing oligonucleotides. The products are focused on treatment of infectious diseases, cancer, metabolic disorders and rare genetic disorders (http://www.santaris.com/product-pipeline; March 14., 2011).

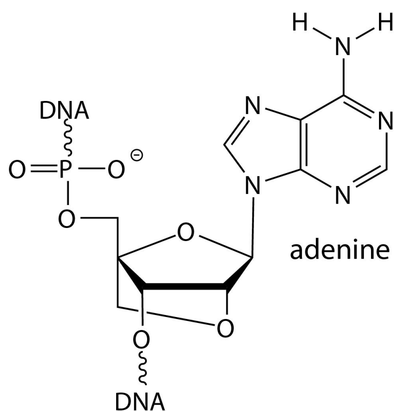

Figure 11
Structure of the adenine LNA nucleotide.

Minor Groove Binders (MGB)

Minor Groove Binders are a group of molecules of which some are added to the buffers, whereas others are covalently linked to oligonucleotides. Here only molecules, which can be covalently linked terminally on the oligonucleotide sequence, are treated. Each MGB molecule composes several similar subunits e.g. N-3-carbamoyl-1,2-dihydro-3H-pyrrolo[3,2-e]indole-7-carboxylate tripeptide, (figure 12) (117). MGB stabilize the anti-parallel duplex helix – especially dA and dT nucleotide rich oligonucleotides - by binding into the minor groove of the double-stranded DNA helix (dsDNA) (20;173). In clinical diagnostics, MGB are often used to improve the hybridization of short oligonucleotides such as PCR primers and TaqMan probes (20;174). When placed on the 5' terminal of PCR primers, MGB increase the efficiency of the PCR reaction and allow the design of shorter PCR primers (174). Placed 3' terminal on TaqMan probes, MGB improve the thermal stability of probe hybridization to single-stranded DNA (ssDNA) targets and decrease the cross-reactivity of single nucleotide mismatches in the seven nucleotides closest to the 3' terminal of the MGB probe (20;155-157). MGB are commercialized by Epoch Biosciences as part of the ELITechGroup and are marketed to allow shorter probes with subsequently improved mismatch discrimination, more universal cycling conditions for probes in multiplex quantitative PCR (qPCR) due to the improved stabilizing effect on adenine and thymine nucleobase rich sequences and improved melting curve analysis because probes are not degraded during PCR (http://epochbio.com/innovation/real_time_pcr_technology/; March 14., 2011).

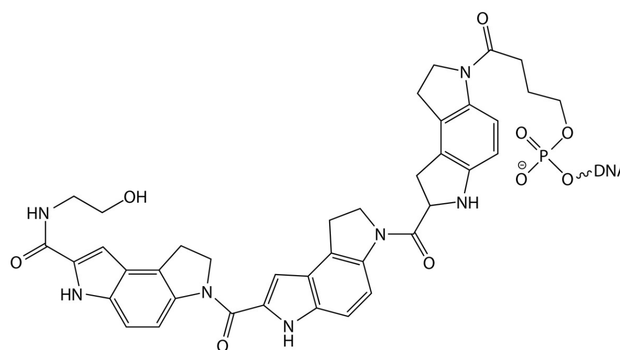

Figure 12
Structure of 3'-MGB

Degradation of the PCR probes is avoided by adding MGB at the 5' terminal of the dual fluorophore labeled probe, thereby protecting the probe against the 5' exonuclease activity of the Taq polymerase. TaqMan hydrolysis probes with 3' terminally placed MGB can be purchased from Applied Biosystems (https://products.appliedbiosystems.com/; March 14., 2011).

Molecules that allow pyrimidine nucleotides in the target nucleotide sequence of triplex helixes

The most important limitation for use of triplex helixes in molecular biology is the need for nucleotide target sequences exclusively formed by purine nucleotides. A number of molecules have been developed to allow pyrimidine nucleotides in the target sequences of triplex helixes (125;175;176). Target nucleotide sequences with a dC nucleotide interruption can be recognized by TFO sequences containing N^4-(6-amino-2-pyridinyl)cytosine (177) or the ^{A}PP (3*H*-Pyrrolo[2,3-*d*]pyrimidin-2(7*H*)-one) (178) nucleobases. Similarly, dT nucleotide interruptions are recognized by TFO sequences containing the S (*N*-(4-(3-acetamidophenyl)thiazol-2-yl)acetamide) nucleobase (179;180). Both thymine to adenine and cytosine to guanine nucleobase pairs are recognized by the D_3 (1-(2-deoxy-β-D-ribofuranosyl)-4-(3-benzamidophenyl)imidazole) nucleobase (176;181) (figure 13).

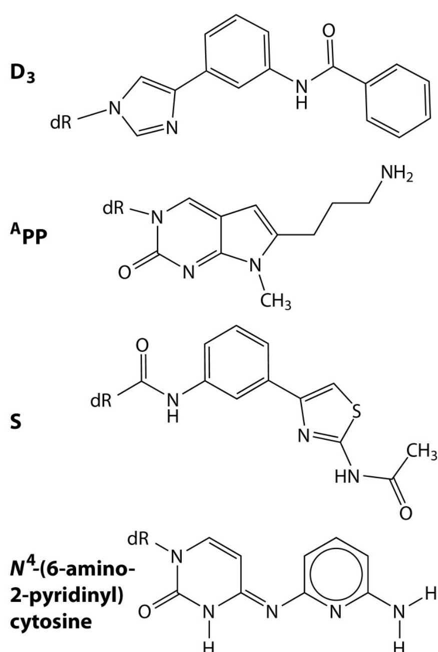

Figure 13
Structure of D_3, ^{A}PP, S and N^4-(6-amino-2-pyridinyl)cytosine. dR is deoxyribose.

Molecules that expand the genetic alphabet by extra nucleobase pairs

A number of molecules have been developed to expand the genetic alphabet by extra base pairs (182). The molecules are either based on formation of base pairs by hydrogen bond formation or solely by stacking interactions (124;183;184). For both types it has been shown that the molecules can be incorporated in PCR based on recognition of hydrogen bond patterns or solely by conformational recognition (185-187). Additionally, the new nucleobases can be used both to stabilize antiparallel duplex helixes and parallel triplex helixes (184;188). The molecules are commercialized like, for example, the Super A™ from Epoch Biosciences that allow for the formation of three hydrogen bonds between the adenine and thymine nucleobases (http://epochbio.com/innovation/superbases/; March 14., 2011).

Molecules designed to remove the pH dependence of cytosine nucleobases in the TFO sequence of parallel triplex helixes

As previously mentioned, triplex helixes are limited by the need of stretches of purine nucleotides in the target sequences. Another limitation is the need for acidic pH to form stable parallel triplex helixes. One alternative approach could be use of antiparallel triplexes instead, however the fact that G-quadruplexes of sequences contain stretches of multiple guanine nucleobases limits this possibility. To allow pH independent parallel triplex helixes, several different molecules have been developed (126;189-192). Use of nucleobase modifications such as 5-methylcytosine (126), 6-oxocytosine (189), 2'-*O*-methylpseudoisocytidine (193;194), pyrazine DDA (188), N^6-methyl-8-oxo-2'-deoxyadenosine (190) and P1 (5-amino-3-methyl-1*H*-pyrazolo[4,3-*d*]pyrimidin-7-one) (192) have eliminated the pH dependence of dC and dT nucleotides based parallel triplex helixes (figure 14). 5-methylcytosine was the first molecule reported to stabilize the pH dependence of dC nucleotides in parallel triplex helixes and is most frequently utilized (126;175). 5-methylcytosine decreases the pH dependence of dC nucleotide in parallel triplex helixes by improving the stacking of the nucleobases in the TFO sequence (191;195;196). TFO sequences containing 5-methylcytosine molecules have been used *in vitro* to both stabilize dC and dT nucleotide based parallel triplex helixes and inhibit transcription (197;198). Parallel triplex helixes modified by 5-methylcytosines are, furthermore, more stable than antiparallel triplex helixes at physiological potassium concentrations (199).

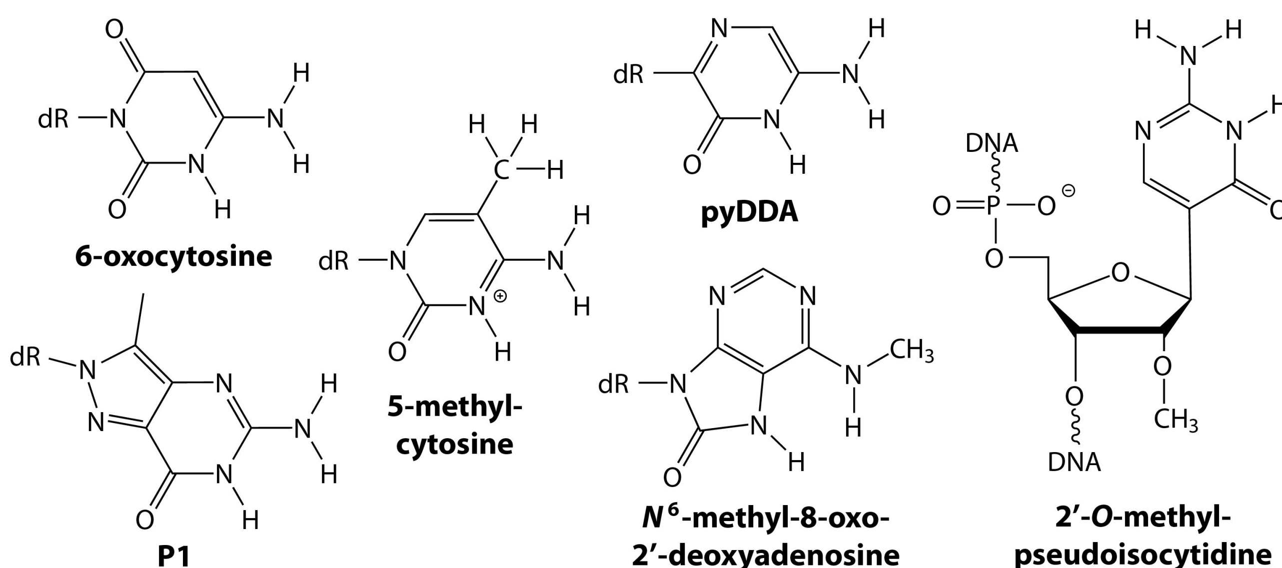

Figure 14
Structure of 6-oxocytosine, P1, 5-methylcytosine, pyDDA, N^6-methyl-8-oxo-2'-deoxy-adenosine and 2'-*O*-methylpseudoisocytidine. dR is deoxyribose.

Peptide Nucleic Acid (PNA)

In the PNA molecule, the phosphate back-bone and furanose moiety are substituted by a *N*-(2-aminoethyl)glycine molecule with the nucleobase attached to the glycine nitrogen by a carbonyl methylene linker (figure 15) (115;200). The back-bone of PNA modified oligonucleotides is neutral, allowing stronger hybridization to DNA or RNA strands, due to the lack of electrostatic repulsion in the back-bone of the oligonucleotide (17;201). Antiparallel duplex helixes between a DNA strand and a PNA modified oligonucleotide forms very wide "P-form" antiparallel duplex helixes with a diameter of 28 Å and 18 base pairs per helix turn (compared with a diameter in B-DNA antiparallel duplex helix formations of approximately 20 Å with 10.5 base pair per helix turn in solution) (202;203). In buffer solutions, PNA modified oligonucleotides are relatively unaffected by ionic strength (17;204), and resistant to nucleases and proteases (205). The Tm of PNA modified oligonucleotides can be predicted by the formula Tm (°C) = 20.79 + 0.83*Tm (by the nearest neighbour model for DNA antiparallel duplex helixes) – 26.13*(fraction of pyrimidines) + 0.44*(nucleotide sequence length) (206). Formation of antiparallel duplex helixes between PNA modified oligonucleotides and DNA or RNA oligonucleotides have been found to be more thermally destabilized (increased mismatch discrimination) by single nucleotide mismatches compared to conventional DNA or RNA antiparallel duplex helixes (17;207). Most PNA modified oligonucleotides have low solubility compared to conventional oligonucleotides that have a tendency to aggregate (208-210). The solubility of PNA modified oligonucleotides decreases for longer oligonucleotides (210;211). PNA modified oligonucleotides can increase Tm of parallel triplex helixes (202;212), more importantly, however, PNA modified oligonucleotides have the ability to invade intact antiparallel duplex helixes (212;213). The qualities of the PNA molecule make PNA modified oligonucleotides especially useful in fluorescence *in situ* hybridization (FISH) (208;214;215), for highly sensitive biosensors (216-219) and for silencing of abundant wild-type targets in PCR based single mutation analyses (220). PNA modified oligonucleotides can be transfected into cells (221-223). The use of PNA modified oligonucleotides for FISH assays are marketed by AdvanDx A/S (http://www.advandx.com/; March 14, 2011).

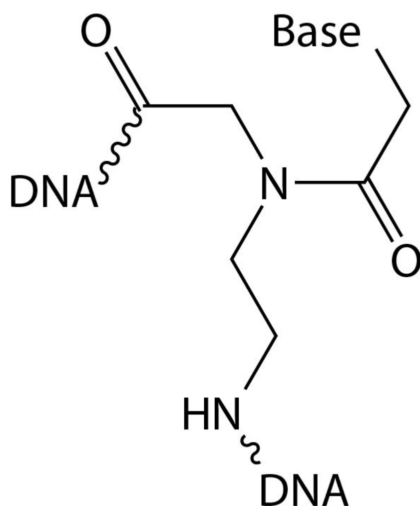

Figure 15
Structure of PNA. Base is one of the nucleobases.

AdvanDx are focused on *in-vitro* diagnostics of species-specific ribosomal RNA from bacteria and yeasts in positive blood cultures.

Phosphoramidate Morpholino Oligonucleotides (PMO)

In PMO molecules, the furanose moiety has been substituted by a morpholino moiety, whereas the back-bone phosphate has been substituted by a phosphoramidate (figure 16) (116). These structural changes eliminate the anionic repulsion in the back-bone of PMO modified oligonucleotides (224;225). PMO molecules were designed to allow antisense applications *in vivo* by improving antiparallel duplex helixes without compromising the cross-reactivity of the oligonucleotides (19;226). PMO modified oligonucleotides are nuclease resistant (227) and the solubility at hydrophilic conditions is good (226;228). Even though PMO molecules were designed for stabilization of antiparallel duplex helixes, they have been found to increase Tm of parallel triplex helixes as well (229). In parallel triplex helixes, PMO modified oligonucleotides have been shown to increase the association rate of parallel triplex helixes significantly (230). PMO modified oligonucleotides are available through Gene Tools LLC (http://www.gene-tools.com/; March 14., 2011).

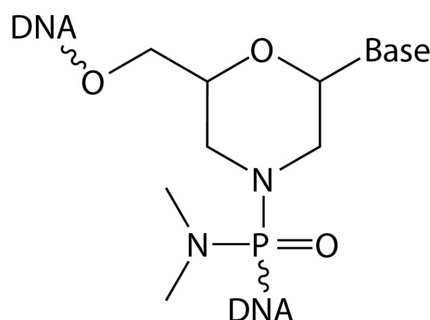

Figure 16
Structure of PMO. Base is one of the nucleobases.

Twisted Intercalating Nucleic Acid (TINA) and AMANY

Para-TINA ((*R*)-1-*O*-[4-(1-pyrenylethynyl)phenylmethyl]glycerol) and AMANY ((*S*)-4-(4-(1H-Phenanthro[9,10-*d*]imidazol-2-yl)phenoxy)butane-1,2-diol) (figure 17) were designed to thermally stabilize parallel duplex and triplex helixes (113;121). Recently, a study on modification of the original TINA molecule showed that the thermal stability of antiparallel duplex helixes was significantly improved by changing the attachment of the ethynylpyrene functional group from *para* to *ortho* ((*R*)-1-*O*-[2-(1-pyrenylethynyl)phenylmethyl]glycerol) (figure 17) (231;232). The effects of different TINA molecule modifications are included in the discussion section. *Para*-TINA molecules have been used to stabilize both antiparallel triplex helixes and G-quadruplexes. Through considered placement of *para*-TINA molecules into a dA and dG nucleotide sequence targeting the human *kras* gene, it was possible to stabilize the G-quadruplex and to induce a strong antiproliferative effect in pancreatic cancer (Pan-1) cells by blocking the *kras* promoter (233). When *para*-TINA molecules were placed differently in the quadruplex forming sequence from the *kras* promoter, it was possible to inhibit potassium-induced TFO self-association into G-quadruplex formations at physiological potassium concentrations (140 mM potassium) (234).

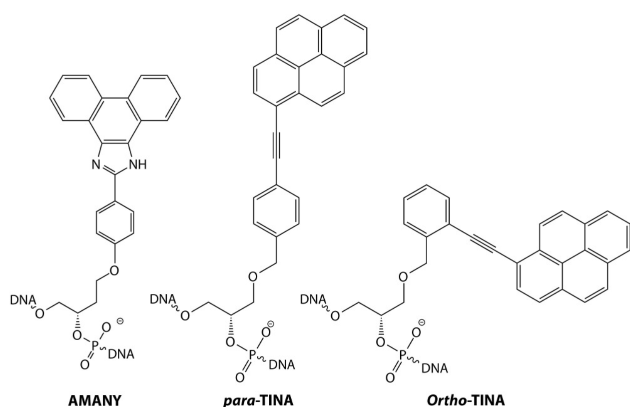

Figure 17
Structure of AMANY, *para*-TINA and *ortho*-TINA

The intellectual property rights for TINA molecules are held by TINA Holding A/S with QuantiBact A/S holding exclusive license to use TINA molecules for *in-vitro* diagnostics of infectious diseases (www.quantibact.com; March 14., 2011), whilst the University of Southern Denmark owns the intellectual property rights for AMANY (http://www.sdu.dk/Om_SDU/Faellesomraadet/ Forskerservice; March 14., 2011).

Zip Nucleic Acid (ZNA)

ZNA is a spermine molecule that allows for conjugation of several spermine molecules to an oligonucleotide sequence (figure 18) (122;235). ZNA modified oligonucleotides stabilize antiparallel duplex helixes by neutralization of the phosphate backbone of each nucleic acid strand prior to hybridization (122). ZNA molecules are covalently linked terminally on the oligonucleotide sequence and stabilize the antiparallel duplex helix by binding in the minor groove (236). Adenine and thymine nucleobase rich oligonucleotide sequences are especially stabilized by spermine molecules due to the narrowing of the minor groove in adenine and thymine nucleobase rich antiparallel duplex helixes (236). In PCR primers, ZNA modified oligonucleotides should be used in buffers with low magnesium concentration to avoid unspecific hybridization and primer-dimer formation due to decreased backbone repulsion between the nucleic acid strands (237). Dual-labeled ZNA modified oligonucleotides used as qPCR probes allow for the shortening of hydrolysis probes and cause lower background fluorescence due to the 3’ quencher's terminal placement on the spermine molecules, thus closer to the 5’ fluorophore (increased signal-to-noise ratio) (238). Initial evaluations of oligonucleotides modified with ZNA molecules have only been done as single-plex reactions and it is unknown whether the tendency of ZNA modified oligonucleotides to hybridize with non-target sequences (increased cross-reactivity) will allow for use in multiplex reactions (237;238). The ZNA molecule is commercialized by Polyplus-transfection SA and oligonucleotides modified with ZNA can be bought for research and *in-vitro* diagnostics through Sigma-Aldrich (since 2009) and Metabion GmbH (since 2010). ZNA™ modified oligonucleotides are marketed as dual-labeled qPCR probes, PCR and RT-PCR primers, for Northern Blot / Dot Blot analysis, for microarrays and for use in In Situ Hybridization assays (http://www.polyplus-transfection.com/technologies/ zna-modified-oligonucleotide/; 14th March 2011).

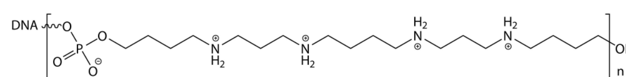

Figure 18
Structure of ZNA.

METHODS AND EXPERIMENTS

Melting point determination on the LightCycler platform

When we initiated our studies of *para*-TINA molecules in 2007, it had been established that *para*-TINA molecules could improve Tm of triplex helixes by placement of *para*-TINA molecules in the TFO sequence (113). Very little was known about the optimal placement, and number, of *para*-TINA molecules (239-241). To be able to deduce design rules for placement of *para*-TINA molecules in parallel triplex helixes and antiparallel duplex helixes, we had to perform a vast amount of Tm determinations. For these studies, we used dC and dT nucleotide based intermolecular parallel triplex helixes in sodium acetate buffer at pH 5.0.

The measurements of several Tm by traditional UV absorbance would have been extremely time and reagent consuming and entailed a lack of precision on the Tm of at least +/-0.5°C. Moreover, the melting peaks from the triplex helix and duplex helix melting could have become superimposed making it difficult to distinguish Tm (242). To overcome these limitations, we used the LightCycler platform for melting curve analysis of triplex helixes as introduced by professor Keith R. Fox's group (242). This LightCycler based approach relies on molecular beacons (243) with fluorescein as the fluorophore. The LightCycler based method has since been used to evaluate the formation of intermolecular dC and dT nucleotide based parallel triplex helixes (175;244).

We found the fluorescence of fluorescein to be pH dependent and to diminish at acidic pH (245;246). With the goal of identifying a useful fluorescence resonance energy transfer (FRET) pair, we evaluated the following donor fluorophores; FAM, HEX, TET, Yakima Yellow, Alexa Fluor488, Rhodamine Green, Chromeo488, ATTO488, DyLight488 and ATTO495, the acceptor fluorophores; Cy5, DyLight594, DyLight633, CALfluor610, CALfluor635, ATTO520, ATTO532, ATTO565, ATTO590, ATTO647N and ATTO680 and the quenchers; BHQ1, BHQ2, BHQ3, EDQ, BB650Q, ATTO540Q and ATTO612Q in sodium cacodylate buffers at decreasing pH. The different fluorophores were selected in an attempt to establish both a single FRET pair for determination of parallel triplex helixes, but also in pursuit of a double FRET system involving a fluorophore on each of the three strands in the triplex helix. In general, ATTO fluorophores were found to be pH insensitive, in contrast to most of the other fluorophores, and performed well on the LightCycler platform. To establish the use of ATTO fluorophores for Tm determinations in pH sensitive parallel triplex helixes and antiparallel duplex helixes, a number of validation experiments were performed. Experiments were conducted in sodium cacodylate, sodium acetate and sodium phosphate buffers due to these buffers' pH only decreasing slightly with increasing temperature. The results from a number of validation experiments were published in the first manuscript using ATTO495 and ATTO647N as the FRET pair (MI). Testing of other ATTO fluorophores as FRET acceptors together with the ATTO495 donor fluorophore revealed that the combination of ATTO495 and ATTO590 fluorophores was an even more efficient and cost-effective FRET pair. As the two FRET pairs performed similarly, the

ATTO495 and ATTO590 FRET pair was used for the melting curve experiments in MII and MIII.

The LightCycler is a carousel-based qPCR platform using air for heating and cooling of thin glass capillaries with a reaction volume from 10 to 100 µL (247). The combination of air based temperature regulation and thin capillaries allows ten times faster cooling and heating compared with microtiter plate based PCR systems (247). The capillary size and temperature control ensured a faster attainment of thermaldynamic equilibrium in the reaction volume (247). The LightCycler2.0 has a blue LED laser, which emits light at 470 nm and six detection channels with filters at 530, 555, 610, 640, 670 and 705 nm. The operational temperature range in the LightCycler2.0 has been restricted to a 37°C to 100°C range. The slowest ramp rate for continuous collection of melting curve data is 3.0°C/minute with one fluorescence measurement per second. The LightCycler2.0 allows the subtraction of a background temperature profile for each fluorophore in the actual buffer before Tm determination. In the LightCycler software, the Tm is determined as the peak of the first derivative. Melting curves collected on the LightCycler are routinely used for evaluation of PCR products and as a tool for genotyping (248;249).

Traditionally, the Tm of nucleic acid structures is defined as the temperature when 50 % of the molecular structures in question are denatured and the remaining 50 % are still annealed (250). For Tm determination, both starting and final fluorescence plateau are identified. The Tm is determined as the median fluorescence between these two plateaus (250). As stated, the LightCycler routinely uses the first derivate method by which the Tm is determined as the peak of the first derivative. The first derivative reflects the temperature when the fluorescence signal changes at the highest rate (250). Tm determined by the two methods differs slightly. A Tm determined by the first derivative method is in general higher than the Tm determined by the median fluorescence method (250). To ensure the accuracy of the melting point determinations, both the dissociation and the annealing curves were used. If dissociation and annealing follow the same reaction kinetics, the Tm determined by the two curves should be the same, and the two curves can be superimposed (250;251). The phenomenon of hysteresis is the difference between the two curves if they cannot be superimposed. Hysteresis is found when the system is not in true thermaldynamic equilibrium or if the dissociation and annealing curves follow different reaction kinetics (250;251).

Capture assay on the Luminex® 200™ platform

In the third manuscript (MIII) we used the Luminex® 200™ platform to evaluate the sensitivity of *ortho*-TINA and *para*-TINA modified oligonucleotides compared to conventional DNA oligonucleotides. The Luminex® 200™ can be described as a modified flow cytometer having two lasers and four detectors.

The Luminex core technology comprises polystyrene microspheres internally loaded with two different fluorescence dyes on a logarithmic scale for differentiation of 100 nonmagnetic or 80 paramagnetic different fluorescence microsphere sets on the Luminex platform (252). The Luminex microspheres are either precoated with short oligonucleotide tags (*x*TAG) or modified with approximately 100 million carboxyl-groups for subsequent coupling to nucleic acids or protein (*x*MAP). The *x*TAG microspheres are used in combination with complementary tags linked by a hexaethyleneglycol (HEG)-spacer to an oligonucleotide sequence of interest. The *x*MAP microspheres can either be conventional polystyrene microspheres with an average diameter of 5.6 µm or paramagnetic polystyrene microspheres (MagPlex™) with an average diameter of 8.6 µm. Detection is performed using biotinylated oligonucleotides or proteins visualized by streptavidin-*R*-phycoerythrin (SA-PE). The excitation of *R*-phycoerythrin has three peaks at 496, 546 and 565 nm with emission maximum at 578 nm. An outline of a Luminex assay for detection of single stranded nucleic acids is shown in figure 19.

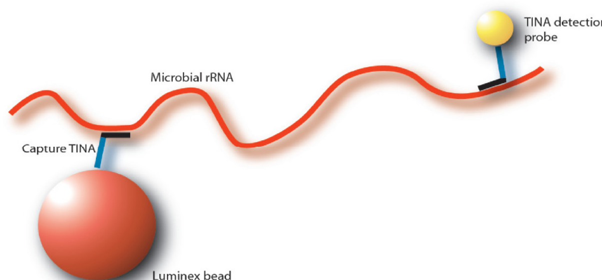

Figure 19
Luminex capture and detection of nucleic acid target

Microspheres are classified in the Luminex by excitation by a “red” laser at 635 nm and the microsphere fluorescence is detected by three avalanche photodiodes (APD). One APD measures side scatter to ensure that only single microspheres are registered, whereas two APD measure fluorescence at 645 to 669 nm and above 712 nm to classify the microspheres. Detection is done by excitation by a “green” YAG laser at 532 nm and the fluorescence is detected by an APD at 563 to 587 nm with linearity over 3.5 Log. To ensure the accuracy of the signal, determination of at least 100 microspheres from each microsphere set should be counted. The fluorescence signal is reported for each microsphere set as the median fluorescence intensity (MFI). The MFI is reported not only to reduce the influence of non-uniformed signal coated microspheres, but also to diminish the influence of microsphere carry-over in the system. Carry-over of smaller fractions of microspheres in the Luminex system is hard to avoid due to the Luminex system design's sample processing. A predefined sample volume is soaked from a 96-well microtiter plate by a sample probe. The height of the probe is adjusted to leave a volume of approximately 20 µL fluid in the well to avoid accidental air leaks into the system. If the sample probe is adjusted to close to the bottom of the well, the microspheres cannot pass unrestricted up into the probe and the sample processing time will be extended or the Luminex® 200™ will fail to count enough microspheres of each microsphere set. The sample volume is processed to the laser chamber in a low salt sheath fluid buffer in plastic tubing with a diameter of 100 µm. The diameter in the laser chamber is narrowed to 10 µm to ensure a continuous flow past the lasers. If air has been leaked into the system, it will normally be trapped at the entrance to the laser chamber together with a number of microspheres. After having passed by the lasers, the sample volume is discharged into a waste container and sheath fluid is poured into the well to cleanse the tubing and probe. When the probe goes down into the sample, it will move up and down several times to try to remove microspheres captured on the outside of the probe, but microsphere carry-over can never be completely avoided.

For target capture purposes amino-modified DNA oligonucleotides are covalently coupled to carboxyl-coated MagPlex™ microspheres following a standard carboiimide (EDC) coupling

procedure (252). Proteins are coupled to Luminex microspheres by a two-step EDC and *N*-hydroxysulfosuccinimide (Sulfo-NHS) coupling procedure (253). Suggestions for standard coupling protocols can be found on the Luminex Corporation's website (www.luminexcorp.com; March 14., 2011). To ensure the accuracy of MFI determination in our assays, we added approximately 2500 microspheres from each microspheres set to an assay and counted 350 microspheres from each microspheres set.

The background fluorescence in a Luminex assay depends on i) which microspheres that are used, ii) the purity of the SA-PE, iii) the hybridization buffer, iv) washing steps in the assay protocol, and v) impurities in the coupling of the oligonucleotides to the Luminex microspheres. Background MFI below 50 is, according to Luminex, acceptable (per personal communication with Luminex), however, we find that background fluorescence generally can be reduced to below a MFI of 10. As the fluorescence dyes in the microspheres are added on a logarithmic scale, a small increase in MFI (from one to nine) is seen for microspheres sets with high concentrations of internal dyes. The background MFI is particularly altered by the choice of SA-PE conjugate and use of additional purified conjugates can almost eliminate the cross-reactivity between the microspheres and the SA-PE conjugates. For in-house nucleic acid protocols, Luminex recommends the use of a 3 M tetramethylammonium chloride (TMAC) buffer with 0.1 % sarkosyl, 4 mM EDTA and 50 mM Tris-HCl at pH 8.0. This buffer reduces the hybridization differences between adenine to thymine and guanine to cytosine nucleobase pairs at the cost of analytical sensitivity (21;254). As an alternative, sodium based buffers can be used. To work as a detection buffer the sodium content of the buffer must be below 400 mM sodium due to sodium concentration above 400 mM changing the detection of microspheres fluorescence in the Luminex system (microspheres are shattered around the bead map).

The size of conventional *x*MAP microspheres has been found to differ significantly with a median diameter of 6.14 µm and a range from 5.6 µm to 12.5 µm compared to Luminex's specifications stated at 5.6 µm +/- 0.2 µm (255). In multiple experiments, we have observed that the MFI decreases across a 96-well microtiter plate. This decrease in MFI is seen for both negative controls and samples. The decrease in MFI is clearly time dependent. When the plate is left in the dark for half an hour before analysis, drift in MFI is eliminated (MIII). Based on these findings, we are convinced that the microspheres sediment within the first 30 minutes. The fraction of microspheres analyzed after a 30 minute incubation is then more uniform in size.

Experimental outline for the three manuscripts

The aim of the first manuscript (MI) was to establish a novel FRET pair for melting curve determinations on the LightCycler™ platform. In the second manuscript (MII), we used the LightCycler™ platform based melting curve method to establish design rules for placement of *para*-TINA molecules in parallel triplex helix forming oligonucleotides. In the third manuscript (MIII), we used the LightCycler™ based melting curve method to evaluate placement of *para*-TINA and *ortho*-TINA molecules in antiparallel duplex helix forming oligonucleotides. Additionally we evaluated the impact of TINA molecules on the analytical sensitivity of a capture assay targeting the *Escherichia coli rrs* gene with the *Pseudomonas aeruginosa rrs* gene as a cross-reactivity control. The experiments reported in the three manuscripts are outlined below (table 1 and 2). Table 1 summarizes all experiments obtained by melting curve determinations by the LightCycler platform based melting curve method, whilst Table 2 covers the experiments from the Luminex platform based experiments. A substantial amount of additional experiments have been performed as assay controls, for establishing and validating the methods, and to ensure that application of these methods would obtain results similar to those already established in the scientific literature.

Table 1

Outline of experiments conducted on the LightCycler™ platform

Objective	Evaluated	Number of Capillaries	Reported in
Evaluation of fluorophores	Four fluorophores at various pH steps	64	MI, fig. 2
Evaluation of FRET pairs	Different FRET pairs for AD and PT at various pH steps	39	MI, fig. 3
Influence of buffer changes and different oligonucleotide concentrations on the ATTO FRET pair	ATTO495 and ATTO 647N FRET pair in AD and in PT at various pH steps	26	MI, fig. 4
Evaluation of different melting curve pro-grams	16 runs in quadruplicate for AD and PT at various pH steps	448	MI, fig 5
Intra-assay variation	Run of 12 capillaries for both AD and PT	24	MI, table 1
Inter-assay variation	Run of 12 capillaries 6 times for both AD and PT	144	MI, table 1
Inter-machine variation	Run of 12 capilaaries on two different machines for AD and PT	48	MI, text
Stability of premixed oligonucleotides	Six runs of six premixed oligonucleotides for both AD and PT	72	MI, test
Stability over time of mixed oligonucleotides	Nine runs of 24 capillaries for both AD and PT with storage at different temperatures	432	MI, fig. 6
Comparision of Tm determined by the UV absorbance method and the LightCycler method	Quadruplicate runs for both AD and PT with and without fluorophores	16 (UV) + 8	MI, fig. 7
Placement of TINA molecules in the TFO of PT	116 TINA modified oligonucleotides and 19 cross-reactivity oligonucleotides	2204	MII, table S1
Placement of TINA molecules in the AD strand of PT	15 TINA modified oligonucleotides and 19 cross-reactivity oligonucleotides	285	MII, table S2
Placement of TINA molecules in both the TFO and AD strand of PT	40 TFO modified with TINA molecules and 15 AD strands modi-fied with TINA molecules	600	MII, table S3
Placement of TINA molecules in shorter oligonucleotides of PT	29 TFO modified with TINA molecules and with different lengths and seven cross-reactivity oligonucleotides	203	MII, table S4
Effect of ramp rate on Tm determinations of PT	28 TFO modified with TINA molecules at two ramp rates	112	MII, table S5
Influence of preincubation time onTm deter-minations of PT	28 TFO modified with TINA molecules at two ramp rates and four different preincubation times	228	MII, table S6
Influence of pH on Tm determinations of PT	28 TFO modified with TINA molecules at two ramp rates and three different pH	168	MII, table S7
Influence of pH and buffer on cross-reactivity	19 cross-reactivity oligonucleotides for PT and AD at different pH and buffer	684	MII, table S8
Placement of *ortho*-TINA molecules and *para*-TINA molecules in AD	50 oligonucleotides modified with *ortho*-TINA and *para*-TINA molecules and 37 cross-reactivity oligonucleotides	1850	MIII, table S1

Table 1. Light Cycler based melting curve determinations in the three manuscripts. AD: antiparallel duplex helix, PT: parallel triplex helix, TFO: triplex forming oligonucleotide, AD strand: antiparallel duplex forming strand of the triplex helix, TM: melting point.

Table 2

Outline of experiments conducted on the Luminex® 200™ platform

Objective	Evaluated	Number of Wells	Reported in
Influence of ionic strengtj on the analytical sensitivity of the assay	Standard curve for two targets with TINA modified oligonucleotides at seven different ionic strengths	1512	MIII, fig. S1
Influence of annealing temperature on the analytical sensitivity of the assay	Seven annealing temperatures with TINA modified oligonucleotides in three different buffers at two target concentrations	1134	MIII, fig. 4
Influence of helper oligonucleotides on the analytical sensitivity of the assay	Standard curve for two targets with TINA modified oligonucleotides with and without helper oligonucleotides	288	MIII, fig. 5

Table 2. Luminex based determination of analytical sensitivity in an antiparallel duplex helix based capture assay with a cross-reactivity control

DISCUSSION OF TINA PROPERTIES AND APPLICATIONS

The family of TINA molecules

A number of studies have been conducted to identify changes in the original *para*-TINA molecule (113) that could improve the thermal stability of parallel triplex helixes (121;231;239-241;256;257). All molecules in the TINA family have the same basic structure with a linker for backbone insertion (L_1) followed by an intercalator (X_1), a new linker (L_2) and a second intercalator (X_2) or schematically written L_1-X_1-L_2-X_2. Table 3 includes the molecules that have been found to improve the thermal stability of parallel triplex helixes compared with the original *para*-TINA molecule, whereas figure 20 includes the diversity of molecules tested (121;231;239-241;256;257). The AMANY molecule and *para*-TINA molecule induce similar increases in thermal stability for parallel triplex helixes (121). Substitution of X_1 with a naphthalene moiety instead of the phenyl moiety of the *para*-TINA molecule (naphthalene-TINA, (*R*)-1-*O*-[4-(1-pyrenylethynyl)naphtalen-1-ylmethyl]glycerol) can improve the thermal stability of a parallel triplex helix by 2 °C compared with the *para*-TINA molecule at similar experimental conditions (table 3 and figure 20) (256). Likewise, a substitution of X_2 with a 2-(dimethylamino)ethyl-naphthalimide moiety instead of the pyrene moiety in the *para*-TINA molecule (naphthalimide-TINA, (*R*)-6-{[4-({ [2,3-Dihydroxypropyl]oxy}methyl)phenyl]ethynyl}-2-[2-(dimethylamino)ethyl]-1H-benzo[de]isoquinoline-1,3(2H)-dione) can induce a similar increase in thermal stability (table 3 and

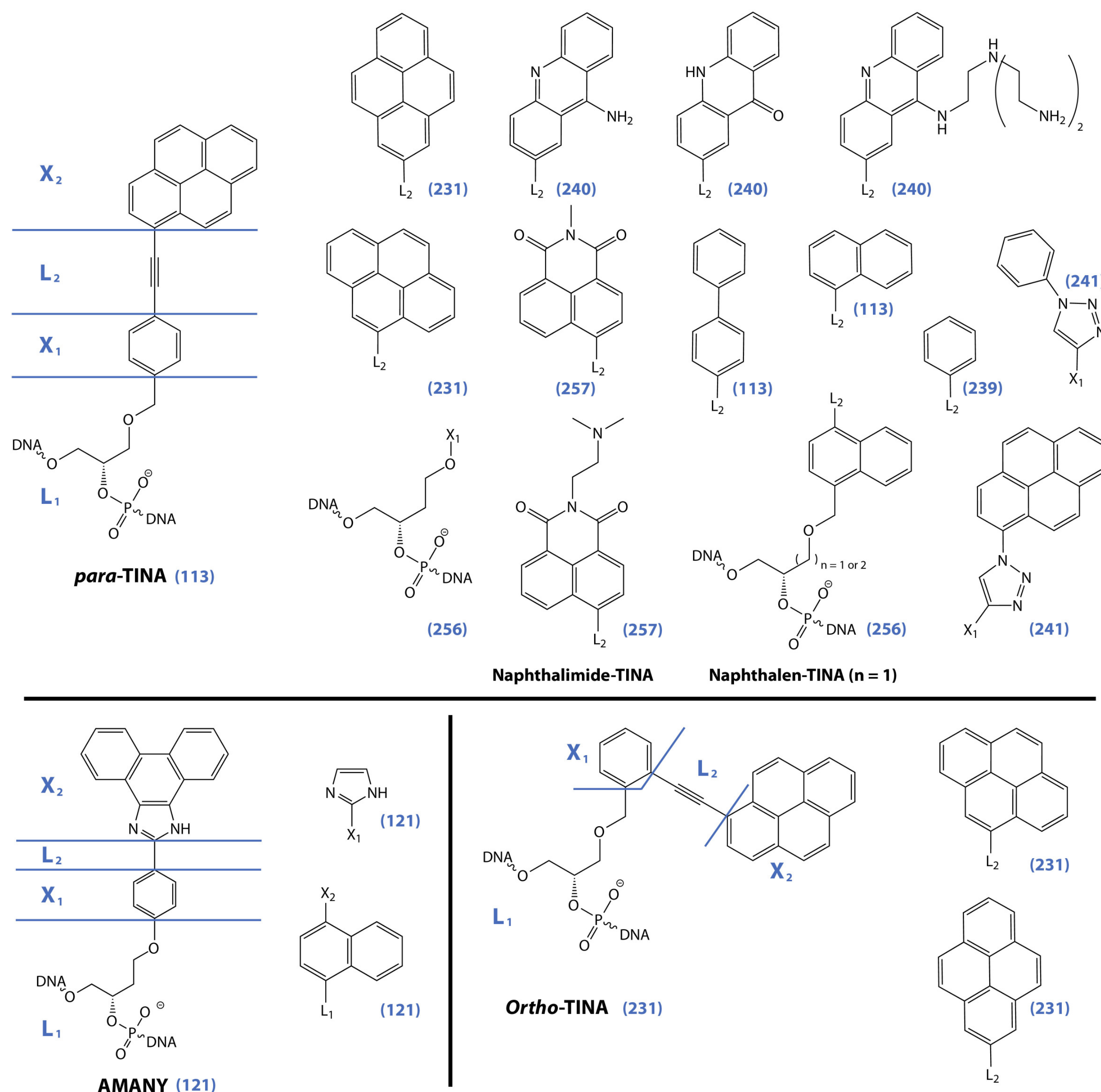

Figure 20

The diversity of structure tested to improve the thermal stabilizing effect of TINA molecules on different nucleic acid helixes. Each molecule with indication of attachment to the basic structure of the TINA molecule and a reference in brackets

figure 20) (257). The naphthalene-TINA and the naphthalimide-TINA molecules' respective influences on thermal stability have only been tested for two modified oligonucleotides by UV absorbance measurement. Uncertainty in UV absorbance measurements and minor differences in experimental conditions may be a contributing factor to the very limited improvements to thermal stability. The remaining molecules presented in figure 20 have not been found to increase the thermal stability of parallel triplex helixes compared to oligonucleotides modified with the *para*-TINA molecule. Thus, despite multiple studies on the structure of TINA molecules for thermal stabilization of parallel triplex helixes, only a few molecules have been identified that improve the thermal stability of parallel triplex helixes to the same extent as the *para*-TINA molecule.

The *para*-TINA molecule was developed solely to increase the stability of parallel triplex helixes and was initially found to decrease the thermal stability of antiparallel duplex helixes (113). Internal placement of *para*-TINA molecules in antiparallel duplex helix forming oligonucleotides decrease the thermal stability, whereas terminal placement of *para*-TINA molecules can improve the thermal stability of antiparallel duplex helixes (table 3). The *ortho*-TINA molecule improves the thermal stability of antiparallel duplex helixes when placed internally, however, the original testing of the *ortho*-TINA molecule's placement did not include placement at the terminal positions of an oligonucleotide sequence (table 3) (231;232). Likewise, AMANY molecules have been found to increase the stability of antiparallel duplex helixes when placed internally in the oligonucleotide sequence. The stabilizing effect of AMANY molecules on antiparallel duplex helixes is increased when the AMANY molecules are placed terminally on the oligonucleotide sequence. The *ortho*-TINA molecule and AMANY molecule both increase the thermal stability of antiparallel duplex helixes, but further studies may identify even better TINA molecules for the stabilization of antiparallel duplex helixes.

Table 3

TINA moleculex afn Tm of parallel triplexes and antiparallel duplex helixes

Triplex forming oligonucleotide (TFO)	PT (pH 6.9) 5’ –ACGGGGAAAGAAAAA 3’ –CTGCCCCTTTCTTTTTT	DNA AD (pH 6.0) 3’ -GGGGAAAGAAAAAA	RNA AD (pH 7.0) 3’ -r(GGGGAAAGAAAAAA)	Reference ID
5’ –CCCCTTTCTTTTTT	27.0/28.0/ 27.5/27.0°C	48.0/49.5/ 48.0°C	52.0°C	(113; 121; 231; 257)
5’ –**X**CCCCTTTCTTTTTT	+17.5/17.5	+5.0		(113; 257)
5’ -**W**CCCCTTTCTTTTTT	+18.0	+.7.0	+7.0	(121)
5’ -**1**CCCCTTTCTTTTTT	+19.5			(257)
5’ -CCC**X**CTTTCTTTTTT	+14.0	-2.5		(113)
5’ –CCCCTT**X**TCTTTTTT	+19,9/17,5/ 18.5/19.0	-1,5/- 1.5°C		(113; 121; 231, 257)
5’ –CCCCTT**W**TCTTTTTT	+18.5	+1.0	+1.0	(121)
5’ –CCCCTT**Z**TCTTTTTT	+10.0	+3.0		(231)
5’ –CCCCTT**1**TCTTTTTT	+21.0			(257)
5’ –CCCCTT**2**TCTTTTTT	+21.0	-2.0		(256)
5’ -CCCCTTT**X**CTTTTTT	+15.5	-3.0		(113)
5’ -CCCCTTTC**X**TTTTTT	+12.5	-3.5		(113)
5’ -CCCCTTTC**W**TTTTTT	+15.5	+1.5	-1.0	(121)
5’ -CCCCTTTCT**W**TTTTT	+20.5	+1.5	+1.0	(121)
5’ -CCCCTTTCTTTTTT**W**	+15.5	+4.5	+3.5	(121)
5’ -**X**CCCCTT**X**TCTTTTTT	+30.0[a]	-1.0		(113)
5’ -CCCCTT**X**T**X**CTTTTTT	+13.0/12.5	-7.0/-7.0		(113;231)
5’ -CCCCTT**Z**T**Z**CTTTTTT	+2.0	-3.0		(231)
5’ -CCCCT**X**TT**X**CTTTTTT	+29.5[a]	-3.0		(113)
5’ –CCCCTT**X**TCT**X**TTTTT	+29.5[a]	-7.0		(113)
5’ -CCCCTT**W**TCT**W**TTTTT	+18.5	-0.5	-2.5	(121)
5’ –CCCCTT**2**TCT**2**TTTTT	+31.5[a]	-5.5		(256)
5’ –CCCCT**2**TTCTT**2**TTTT	+31.5[a]	-3.0		(256)

X = *para*-TINA ((*R*)-1-*O*-[4-(1-pyrenylethynyl)phenylmethyl]glycerol) (figure 17 and figure 20)
W = AMANY ((*S*)-4-(4-(1H-Phenanthro[9,10-d]imidazol-2-yl)phenoxy)butane-1,2-diol) (figure 17 and figure 20)
Z = *ortho*-TINA ((*R*)-1-*O*-[2-(1-pyrenylethynyl)phenylmethyl]glycerol) (figure 17 and 20)
1 = naphthalimide-TINA ((*R*)-6-□[4-(□ [2,3-Dihydroxypropyl]oxy□methyl)phenyl]ethynyl□-2-[2-(dimethylamino)ethyl]-1H-benzo[de]isoquinoline-1,3(2H)-dione)) (figure 20)
2 = naphthalene-TINA ((*R*)-1-*O*-[4-(1-pyrenylethynyl)naphtalen-1-ylmethyl]glycerol) (figure 20)

Conditions for UV-absorbance measurements in references (113;121;231;256;257):
PT (parallel triplex helix): 1.5 µM TFO + 1.0 µM dsDNA in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 6.0. [a]: Third strand and duplex melting overlaid.
DNA AD (antiparallel duplex helix) (pH 6.0): 1.0 µM of each oligonucleotide in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM MgCl2, pH 6.0
RNA AD: 1.0 µM of each oligonucleotide in 10 mM sodium phosphate, 140 mM NaCl, 1 mM EDTA, pH 7.0.
DNA AD (pH 7.0): 1.0 µM of each oligonucleotide in 10 mM sodium phosphate, 140 mM NaCl, 1 mM EDTA, pH 7.0.

Thermal stability studies of TINA modified oligonucleotides

As described in methods and experiments, we used the LightCycler2.0 platform based method to determine several thousand Tm for placement and number of TINA molecules in parallel triplex helixes and antiparallel duplex helixes. Based on these data, we were able to deduce a number of simple and reproducible design rules for placement of TINA molecules in parallel triplex helixes and antiparallel duplex helixes (MII and MIII). As previously mentioned we initially used an ATTO495 and ATTO647N fluorophore FRET pair (MI), however, this was subsequently replaced by the ATTO495 and ATTO590 fluorophore FRET pair (MII and MIII). This was done after the initial validation experiments indicated that the two FRET pairs performed equally well, but the second FRET pair had higher FRET efficiency and was more cost-effective.

LightCycler platform based Tm determinations and hysteresis

Design rules for placement of TINA molecules in parallel triplex helixes were determined in sodium acetate buffer at pH 5.0. A 19 nucleotide TFO sequence was used in combination with two oligonucleotides forming a 23 base pair antiparallel duplex helix. Tm determinations, dissociation and annealing curves were collected at a ramp rate of 3°C/minute. By these settings hysteresis between the dissociation and annealing curves was observed and could not be eliminated at a ramp rate of 0.2°C/minute. When shorter oligonucleotides were used in combination with increasing pH, the hysteresis could be eliminated at the slower ramp rate (MII).

The observed hysteresis can be explained, since dissociation of triplex helixes can occur by following three different patches; i) dissociation of the TFO sequence from the triplex helix first, and thereafter dissociation of the antiparallel duplex helix at higher temperature (predominately) (258;259), ii) a simultaneous dissociation of all three nucleotide strands in the triplex helix (as observed in MII) (48;258-260), and iii) in rare cases at low pH a dissociation of the antiparallel duplex forming strand of the parallel triplex helix first, followed by dissociation of the parallel duplex helix at higher temperature (111;261). The *de novo* formation of the triplex helix is based on the formation of an antiparallel duplex helix first, followed by annealing of the TFO sequence to the antiparallel duplex helix. At standard conditions, the parallel triplex helixes modified with TINA molecules were more stable than the underlying duplex helixes, which led to a simultaneous dissociation of all three strands in the parallel triplex helix. When shorter TFO sequences and increasing pH were used, the stability of the triplex helix decreased. As a consequence, the TFO strand dissociated from the underlying antiparallel duplex helix at a temperature below the Tm of the underlying antiparallel duplex helix. Therefore, in conclusion, at standard conditions the dissociation and annealing of the TINA modified parallel triplex helixes followed different reaction kinetics and hysteresis was observed. The hysteresis was eliminated when the kinetics of the dissociation reaction were changed. Subsequently, we validated that the differences in dissociation Tm at faster and slower ramp rates were parallel shifted for both perfect matching oligonucleotides and oligonucleotides with one mismatch. Additionally, it was validated that the number or the placement of TINA molecules did not influence the parallel shift in dissociation Tm at different acidic pH levels. Since the parallel shift was uniform, we concluded that the Tm determined at the faster ramp rate reflected the Tm determined at the slower ramp rate – with a similar overestimation of Tm in the different experiments at the faster ramp rate. Based on these observations, we conducted the study on parallel triplex helixes at the faster ramp rate.

The study of *para*- and *ortho*-TINA molecules for thermal stabilization of antiparallel duplex helixes was conducted at a ramp rate of 3°C/minute. Dissociation and annealing curves could be superimposed and no hysteresis was observed in any of the antiparallel duplex helix experiments (MIII). These observations reflect that the reaction kinetics for formation of antiparallel duplex helixes are significantly faster than the reaction kinetics of the formation of triplex helixes (57-61).

Tm of TINA modified oligonucleotides in parallel triplex helixes and antiparallel duplex helixes

In the study of parallel triplex helixes with *para*-TINA molecules, we established a number of design rules for placement and number of *para*-TINA molecules (MII). In general, TINA molecules should be placed as a bulge insertion in the TFO sequence. Two TINA molecules in the TFO sequence should always be placed with at least three to four nucleotides in-between. At pH below 6.0, it was beneficial to place a *para*-TINA molecule at each end of the TFO sequence. Additional *para*-TINA molecules should be placed for each half (5 to 6 nucleotides) or whole helixturn (10 to 11 nucleotides). When pH is above 6.0, the *para*-TINA molecule should be placed at the centre of the TFO sequence. Additional *para*-TINA molecules should still be placed for each half or whole helixturn towards both ends of the TFO sequence. When the TFO sequence length was shorter than nine nucleotides, a *para*-TINA molecule should be placed at each end with no internal *para*-TINA molecules. Placement of *para*-TINA molecules opposite each other in the TFO sequence and in the antiparallel duplex strand of the parallel triplex helix induced in all observed cases a thermal destabilization of the parallel triplex helix. These observations are likely to be due to competition between the two *para*-TINA molecules for the same position in the nucleobase stacking of the parallel triplex helix.

The design rules for placement of TINA molecules in antiparallel duplex helixes were similar (MIII). Both *para*- and *ortho*-TINA molecules increased Tm when placed terminally on the oligonucleotide sequences. When placed internally, the stabilizing effect of *ortho*-TINA molecules decreased towards the centre of the oligonucleotide – however with a general increase in Tm. *Para*-TINA molecules largely reduced the Tm when placed internally, but the destabilizing effect was accentuated towards the centre of the oligonucleotide. If placed internally, both *ortho*- and *para*-TINA molecules should be placed with a half or whole helix turn in-between.

Our Tm results on placement of TINA molecules are in concordance with the few results obtained in the structural studies of TINA molecules (table 3) (121;231;239-241;256;257). Interestingly, the Tm results collected for INA molecules are also in concordance with our design rules (123;141;262). We therefore speculate that the Tm stabilizing effect of different intercalator molecules in DNA helixes may follow similar design rules.

ΔTm of TINA modified oligonucleotides in parallel triplex helixes and antiparallel duplex helixes

Nucleobase mismatches in the target strand of parallel triplex helixes have been found to increase the dissociation speed of the triplex helix, but have not been found to influence the annealing of the triplex helix (MII) (54). By using dissociation curves for Tm determination of parallel triplex helixes we consistently observed ΔTm to be higher in parallel triplex helixes compared to antiparal-

lel duplex helixes. These studies were performed in similar buffers, but also in buffers that ensured the stringency of both antiparallel duplex helixes and parallel triplex helixes. Increased ΔTm in parallel triplex helixes compared to antiparallel duplex helixes are in concordance with previous studies (51;52). As expected, we found that a nucleobase mismatch should be placed opposite a nucleobase in the centre of the TFO sequence to ensure the highest ΔTm (53). When a TINA molecule was intercalating directly 3’ or 5’ to the nucleobase mismatch, the ΔTm of parallel triplex helixes and antiparallel duplex helixes were reduced (MII and MIII). This effect was eliminated when the TINA molecule was intercalating two or more nucleobases apart from the mismatch nucleobase. In the ΔTm study of INA molecules, a similar effect for INA molecules could be found in the data set (141). In general, the masking effect of *para*-TINA molecules was more pronounced than for *ortho*-TINA molecules. This effect on ΔTm by molecules intercalating directly adjacent to the mismatch is likely to constitute a general rule for molecules that intercalate in nucleic acid helixes.

Generalization of design rules for placement of TINA molecules in triplex helixes and antiparallel duplex helixes

To assess the applicability of the presented design rules for TINA molecules' placement in oligonucleotides participating in formation of triplex helixes and antiparallel duplex helixes, it is necessary to consider whether the stabilizing effect on oligonucleotide Tm by TINA molecules is i) sequence independent, ii) changed by oligonucleotide length, and iii) influenced by the fluorophores in the FRET pair. The first question is of utmost importance and will be addressed in section 4.3.1 to 4.3.3. The question of whether the stabilizing effect of TINA molecules on oligonucleotides was changed by oligonucleotide length was partly addressed in MII and MIII. In both manuscripts, we found the stabilizing effect of TINA molecules increased with shortening of the nucleotide sequence. The increased stabilizing effect of TINA molecules on shorter oligonucleotides in triplex helixes and antiparallel duplex helixes was expected and can be modulated by assay stringency, however, it may also be sensitive to changes in the oligonucleotide sequence (174). As both manuscripts are based on a single target oligonucleotide sequence, the question regarding nucleotide sequence's influence on oligonucleotides' nucleic acid helix stability, with varied nucleotide lengths, cannot be answered at present. The fluorophores may also influence the stability of the

nucleic acid helix by stacking interactions with the nucleic acid helix (MI). In our studies, the fluorophores were placed on linkers and were always placed at the same position. Due to this, the fluorophores would be likely to improve the stability of the nucleic acid helix to the same extent independently of the oligonucleotide sequence.

Applicability of design rules for placement of TINA molecules in parallel triplex helixes

In the Tm study on parallel triplex helixes, we focused on the position and number of *para*-TINA molecules in the TFO sequence (MII). This study was based on a single target sequence that allowed for comparison of the complete set of *para*-TINA modified TFO sequences and *para*-TINA modified antiparallel duplex strands of the parallel triplex helix as well as further evaluation of single mismatches in the target oligonucleotide sequence. The selected target nucleotide sequence was also used to determine *para*-TINA molecules placed in shorter TFO sequences' influence on Tm. Studies of dC and dT nucleotide based parallel triplex helixes have shown that the Tm of a dC and dT nucleotide based parallel triplex helix is stabilized by increasing numbers of C^+·G:C deoxytriplets at pH below 7.0 and that alternating dC and dT nucleobases stabilize the Tm of the parallel triplex helix the most (47-50). Table 4 illustrates the penalty of adjacent cytosine or thymine nucleobases in dC and dT nucleotide based parallel triplex helixes. To evaluate the effect of *para*-TINA molecules in different dC and dT nucleotide based parallel triplex helixes, we performed a pilot study (table 5) with ten different *para*-TINA molecules containing TFO sequences (the sequences were five nucleotides shorter than the conventional DNA TFO sequences). In general, *para*-TINA molecules did not change the Tm relation between the ten TFO sequences. Shortening TFO sequences from 17 to 12 nucleotides stabilized some of these sequences more than others of the same type. In all cases, the TFO sequence was stabilized if a run of adjacent dC or dT nucleotides was deleted (table 5). In conclusion, we find that the design rules from the dC and dT nucleotide based parallel triplex helix study is applicable to other dC and dT nucleotide based parallel triplex helixes.

Table 4

Tm of parallel triplex helixes with different concentrations of C^+·G:C deoxytriplets

			Y repeats of X identical bases						
TFO sequence	C%	TM (°C)	X = 2	3	4	5	6	7	9
ATTO590-CCCCCCCCCTCCCCCCC	94	*	Y =					1	1
ATTO590-TCCCCCTCCTCCCCCCT	76	*	1			1	1		
ATTO590-TCTCCCTCCTCTCTCCC	65	71.9	1	2					
ATTO590-TCTCCTTCCTCTCTCTC	53	75.4	3						
ATTO590-TCTCTTTCTTCTTT CTT	53	74.7	1	2					
ATTO590-TCTCCTTCCTCTCCTTC	53	72.6	5						
ATTO590-TCCCTTTTCCCCTTTCC	53	65.9	1	2	2				
ATTO590-CCCTTTTTCCCCCCTTT	53	62.1		2		1	1		
ATTO590-TCTCCTTCTTCTCTCTT	41	74.2	4						
ATTO590-TCTCTTTCTTCTTTCTT	29	72.9	2	2					
ATTO590-TCTTTTTCTTTTTTCTT	18	59.4	1			1	1		
ATTO590-TTTTTTTCTTTTTTTTT	6	52.2						1	1

* No melting curves (probably due to G-quadruplex formation in the target strand). Experimental conditions were as described in MII. In short, 1µM of each oligonucleotide were measured in 50 mM sodium acetate buffer with 100 mM sodium chloride and 10 mM magnesium chloride at pH 5.0 at a ramp rate of 3°C/minute.

Table 5

Tm of different dC and dT nucleotide based parallel triplex helixes modified with *para*-TINA molecules

				Y repeats of X identical bases		
TFO	ID	TFO sequence	Tm (°C)	X = 2	3	4
DEC	LC023	ATTO590-XTTCTCTXTCCTCTX	73.4	Y = 2		
DEC	LC026	ATTO590-XTCTCCTXTCTTCTX	71.7	2		
DEC	LC025	ATTO590-XTCTTCTXTCTCCTX	71.5	2		
DEC	LC024	ATTO590-XTTCCTTXTCCTCTX	71.2	4		
DEC	LC028	ATTO590-XTCCTCTXTCCTTTX	70.5	2	1	
DEC	LC029	ATTO590-XTCCCTTXTCTTCTX	70.3	2	1	
DEC	LC027	ATTO590-XTCCTCTXTTTCCTX	70.3	2	1	
DEC	LC022	ATTO590-XTTTCCTXTCTCCTX	70.0	2	1	
DEC	LC030	ATTO590-XTTTCCTXTCCCTTX	68.1	2	2	
DEC	LC021	ATTO590-XTTTTCTXTCCCCTX	65.5			2
DEC	LC036	ATTO590-TCTCCTTCTTCTCTCTT	74.6	4		
DEC	LC033	ATTO590-TTCTCTTCCTCTTTCTC	73.6	3	1	
DEC	LC034	ATTO590-TTCCTTTCCTCTCTTCT	73.1	4	1	
DEC	LC035	ATTO590-TCTTCTTCTCCTTTCCT	70.8	4	1	
DEC	LC038	ATTO590-TCCTCTTCCTTTTCTCT	70.5	3		1
DEC	LC039	ATTO590-TCCCTTTCTTCTTCCTT	70.5	4	2	
DEC	LC037	ATTO590-TCCTCTTTTCCTCTTTC	70.1	2	1	1
DEC	LC040	ATTO590-TTTCCTTCCCTTTCTTC	68.5	3	3	
DEC	LC032	ATTO590-TTTCCTTCTCCTTTTCC	67.9	4	1	1
DEC	LC031	ATTO590-TTTTCTTCCCCTCCTTT	65.7	2	1	2

Table 5. Experimental conditions were as described in MII. In short, 1 µM of each oligonucleotide were measured in 50 mM sodium acetate buffer with 100 mM sodium chloride and 10 mM magnesium chloride at pH 5.0 at a ramp rate of 3°C/minute.

Applicability of design rules for placement of TINA molecules in antiparallel triplex helixes

Antiparallel triplex helixes with *para*-TINA molecules have not been systematically investigated. Only two studies have been published that characterize *para*-TINA containing oligonucleotides in antiparallel triplex helixes (234;263). In a study of nine *para*-TINA molecules containing dG and dT nucleotide based antiparallel triplex helixes, the optimal TFO sequence was found to be a sequence containing two *para*-TINA molecules with six nucleotides in-between (263). This indicates that placement of *para*-TINA molecules in antiparallel triplex helixes may follow the same design rules as for dC and dT nucleotide based parallel triplex helixes, however, specially designed studies are needed.

In the study regarding placement of *para*-TINA molecules in antiparallel triplex helixes, a parallel triplex helix based on dG and dT nucleotides in the TFO sequence was included (263). Parallel triplex helix formations with dG and dT nucleotides in the TFO sequence are only formed if longer sequences of the same nucleotide (dG or dT) are present in the TFO sequence and are favoured by non-stringent buffer conditions (70;264-266). The destabilizing effect of alternating dG and dT nucleotides in the TFO sequence of parallel triplex helixes is caused by distortion of the backbone of the oligonucleotide (267;268). Subsequently, TFO sequences based on alternating dG and dT nucleotides and TFO sequences with shorter sequences of the same nucleotide form antiparallel triplex helixes, whereas TFO sequences consisting strictly of longer sequences of either dG or dT nucleotides form parallel triplex helixes (267;268). Using our FRET based LightCycler2.0 method and standard TFO sequence from MII, we have determined Tm of dC and dT nucleotide based parallel triplex helixes and dT and dG nucleotide and dA and dG nucleotide based antiparallel triplex helixes. However, we have not succeeded in collecting melting curves for dG and dT nucleotide based parallel triplex helixes (unpublished data).

Applicability of design rules for placement of TINA molecules in antiparallel duplex helixes

The design rules for placement of TINA molecules in oligonucleotides participating in formation of antiparallel duplex helixes are based on a single oligonucleotide target sequence. We found that TINA molecules should be placed terminally at each end of the oligonucleotide sequence for maximum increase of Tm of an antiparallel duplex helix. If placed internally, the TINA molecules should be placed with a number of nucleotides equalling a half or whole helix turn in-between (MIII). Terminal placement at each end of the oligonucleotide leads to the stabilizing effect of TINA molecules being less prone to sequence dependence because the TINA molecules will only stack at the ends of the nucleobase structure. The stabilizing effect of TINA molecules at the end of the oligonucleotide sequence may still be minimized by higher percentages of cytosine and guanine nucleobases in the terminal sequences of the oligonucleotide structure, however, further studies are needed to clarify this. Despite the use of only one single nucleotide sequence in the Tm study, we have tested a number of different oligonucleotide sequences on the Luminex®200™ platform as part of progressing application studies of TINA molecules containing oligonucleotides. Using a number of different target sequences, we have not observed any sequence dependence in the stabilizing effect of TINA molecules when placed terminally on the ends of the target oligonucleotides (unpublished data).

Interestingly, the Tm results observed for INA molecules are in concordance with our design rules for placement of TINA molecules in antiparallel duplex helixes (123;141;262). The stabilizing effect of TINA molecules placed terminally on the oligonucleotide sequence fits well with previous studies investigating the stabilizing effect of different aromatic molecules when placed terminally at the ends of double strand DNA helixes (23;133). We, therefore, speculate again that the Tm stabilizing effect of different interca-

lator molecules in DNA helixes may follow similar design rules even though further studies are needed to decisively address such a hypothesis.

Assay conditions and the use of ortho-TINA molecules in antiparallel duplex helixes on the Luminex®200™ platform

The simple design rules for placement of TINA molecules in antiparallel duplex helixes were applied to oligonucleotide design on the Luminex®200™ platform (MIII). Both *para*-TINA and *ortho*-TINA modified oligonucleotides improved the analytical sensitivity of capture of a biotinylated *Escherichia coli rrs* PCR product on the Luminex®200™ platform. At stringent conditions, a 27-fold improvement in analytical sensitivity was observed using *ortho*-TINA modified oligonucleotides. For *para*-TINA modified oligonucleotides, a seven-fold increase in analytical sensitivity was observed, and even at 1 Molar ionic strength oligonucleotides modified with *ortho*-TINA molecules or *para*-TINA molecules were able to increase the analytical sensitivity four-fold. Results were obtained with two different sets of oligonucleotides and the assay conditions were chosen to allow for mismatch discrimination towards the corresponding genetic sequence from *Pseudomonas aeruginosa*. The nucleotide sequence from the *Pseudomonas aeruginosa rrs* gene was chosen, since it is the most closely related known sequence among human pathogenic microorganisms. The Tm data obtained on the LightCycler2.0 platform showed that oligonucleotides modified with *para*-TINA molecules at the 5' and/or 3' terminal positions increased the Tm more than the corresponding *ortho*-TINA modified oligonucleotides. This is in contrast to the competitive annealing capture assay on the Luminex®200™ platform. Based on the Luminex data, it seems likely that the capture hybridization of *para*-TINA modified oligonucleotides is slower than the hybridization by *ortho*-TINA modified oligonucleotides entailing the improved analytical sensitivity by *ortho*-TINA modified oligonucleotides in the Luminex®200™ based capture assay.

Using the standard one-step EDC based microspheres coupling procedure as suggested by Luminex Corporation, we observed lower coupling efficacy of TINA modified oligonucleotides to the MagPlex™ microspheres than for conventional DNA oligonucleotides. This was solved using a two-step EDC and sulfo-NHS based coupling procedure. We believe that the longer coupling procedure might be necessary to allow packing of TINA modified oligonucleotides on the polystyrene microspheres' surface.

Competitive capture of denatured PCR product on the MagPlex™ microspheres had to be optimized to ensure the capture of conventional DNA oligonucleotides at stringent conditions. The placement on ice turned out to be of utmost importance (269;270). In the final procedure, we denatured the PCR product and placed it on ice before the addition of cooled hybridization buffer. This was necessary to decrease the reannealing of PCR product before incubation. If hybridization buffer was added before denaturation, the PCR product would reanneal before it could be cooled sufficiently to allow annealing to the oligonucleotides on the MagPlex™ microspheres. This was especially pronounced as the stringency of the hybridization buffer was decreased.

Use of nucleic acid stabilizing molecules to improve the stability of antiparallel duplex helixes for clinical diagnostics and therapy

As mentioned in the introduction, point-of-care diagnostics are desirable to improve clinical diagnostics and patient management. At present, the most promising point-of-care technologies are based on target amplification and signal amplification systems like the Verigene® System from Nanosphere Inc., the FilmArray™ Instrument from Idaho Technology, the GeneXpert® System from Cepheid and the BD MAX™ System from BectonDickinson.

Conjugation of MGB or ZNA to the 5'-end of PCR primers allow for use of shorter PCR primers (174;237), whereas PNA and LNA modified PCR primers can be used to block abundant wild-type targets thereby allowing the amplification of rare targets by DNA nucleotide based PCR primers (164;220). LNA, MGB and ZNA are used to improve the hybridization of qPCR TaqMan probes to the target nucleotide sequence and allow the shortening of the qPCR TaqMan probes (20;157;238). Shortening of the qPCR TaqMan probes is beneficial due to the discrimination of single nucleobase mismatches increasing with reduction of oligonucleotide sequence length (20).

In hybridization based assays such as microarrays and biosensors (153;216;218), LNA and ENA containing oligonucleotides are especially beneficial for capture of RNA targets in liquid solutions, whereas PNA modified oligonucleotide probes are also used for *in-situ* FISH based capture of bacterial and fungal ribosomal RNA targets from positive blood cultures (215). PNA, PMO, ENA and LNA modifications of antiparallel duplex helix forming oligonucleotides all improve the nuclease resistance of the oligonucleotides (120;152;205;227). At present, LNA modified oligonucleotides, in particular, are utilized in initial studies for development of antisense therapy.

Counteracting the limitations of triplex helixes by nucleic acid stabilizing molecules

Since the discovery of nucleic acid triplex helixes in 1957 (36), a substantial number of studies have been conducted on triplex helixes. Despite such efforts, the commercial use of triplex helixes in clinical diagnostics and therapy is almost non-existing. A number of different applications of triplex helix forming oligonucleotides have been proposed but they are not currently used as standard procedures in molecular biological research, diagnostics nor therapy. This may be explained by the natural limitations of triplex helixes. The following sections discuss some of the proposed applications of triplex helixes and some of the molecules that have been developed to solve the limitations of triplex helixes.

Use of triplex helix forming oligonucleotides in diagnostic assays

Triplex helix forming oligonucleotides have been introduced in diagnostic assays for i) purification and isolation of intact dsDNA (271-273), ii) visualization of dsDNA (274-276), and iii) development of biosensors based on formation of triplex helixes (277-279).

Purification and isolation of dsDNA by triplex forming oligonucleotides is an evident application of triplex forming oligonucleotides in diagnostic assays. Both dC and dT nucleotide based parallel triplex helixes and dG and dT nucleotides based antiparallel triplexes have been introduced (271;273). A major concern regarding the present method is the lack of cross-reactivity controls in the studies. The cross-reactivity of the oligonucleotides was only tested for one unrelated polypurine target sequence showing cross-reactivity of dC and dT nucleotide based parallel triplexes at acidic pH on polystyrene surfaced magnetic beads (273). Polystyrene surfaced magnetic beads (Dynabeads® M-280 Streptavidin from Invitrogen) are prone to non-specific binding, which increases as pH decreases (273;280). Furthermore, studies have been conducted in buffers with high salt concentrations

thereby producing uncertainty about the ability of the method to discriminate oligonucleotide mismatches (271-273). In a single study, a dG and dT based antiparallel triplex forming oligonucleotide was included (273). The selected oligonucleotide sequence contains four streches of three or four dG nucleotides and is likely to allow formations of G-quadruplexes, which has not been addressed in the publication (273). At present, the method for sequence-specific purification and isolation of dsDNA by triplex helix forming oligonucleotides needs optimization to ensure: i) faster kinetics for formation of the triplex helixes, ii) no cross-reactivity to closely related nucleotide sequences whilst iii) simultaneously maintaining a high analytical sensitivity.

Visualization of dsDNA by a TFO has been achieved by molecular beacons (274;275) and by third-strand *in situ* hybridization (TISH) (276). In the visualization of dsDNA by TISH, a 5-methylcytosine and dT nucleotide based parallel triplex helix forming oligonucleotide was used and shown to be sequence specific whilst enabling discrimination of a single dA to dG transition in the target sequence (276). The feasibility of the molecular beacon strategy was shown by using a dG and dT based antiparallel triplex helix forming oligonucleotide (275). Visualization of dsDNA by TFO sequences has thus been demonstrated, but is at the present not commercialised.

Capture of dsDNA on biosensors by triplex forming oligonucleotides is currently under development. Recently, the capture of a conserved polypurine tract in HIV-1 strains by dG and dA nucleotide based antiparallel triplex helixes or dC and dT based parallel triplex helixes was reported and the biosensor was shown to allow for detection of approximately 10 nM of dsDNA target direct from blood serum (279). Cross-reactivity was tested for the dC and dT based parallel triplex helix forming oligonucleotide using a 15 nucleotide sequence with four nucleotide mismatches and high concentrations of the double stranded target DNA sequence (279). No cross-reactivity was detected at these conditions. The present methods work well as a qualitative method but needs improvement in linearity to allow for quantification of targets. Allowing for quantification of targets by this method will probably be difficult due to the method being based on measurement of signal reduction in percentages by increasing target concentrations.

Use of triplex helix forming oligonucleotides for *in vitro* cellular systems and for *in vivo* therapy

A number of different applications of TFO sequences for manipulation of dsDNA in cellular systems have been established (reviewed in (281;282)). TFO sequences have been introduced to allow for inhibition (283-285) and activation of transcription initiation (286-288), termination of target elongation (289), site-specific mutagenesis (290;291), third strand mediated cleavage of dsDNA (292-296) and site-specific recombination of genes (297-300). TFO's ability to induce mutations adjacent to the target site is a potential limitation for its use in human *in vivo* therapy (290), especially when TFO are introduced for gene control. One solution to this problem will be to design TFO to target nucleotide sequences upstream from the transcription start site, thereby limiting the mutation rate in coding regions. Fortunately, triplex helix forming sequences in the human genome are frequently located in promoter sequences (66).

Use of TFO for antigene therapy *in vivo* is desirable to allow for gene-specific treatment of diseases. Antigene therapy promises the possibility to limit the number of adverse effects of the treatment because only a specific gene is targeted by the treatment. Additionally, only a few triplex helix forming oligonucleotides are needed per cell compared with antisense, RNA interference and extracellular aptamer treatment strategies, in which multiple RNA or protein target molecules have to be regulated at a given time (301;302). Using fewer oligonucleotides limits the possibilities for toxicity problems due to large quantities of a specific oligonucleotide (302;303). As in all oligonucleotide based *in vivo* treatment strategies, both the delivery of the oligonucleotides across the cell membrane and the stability of the oligonucleotides against degradation by intracellular nucleases are challenges that need to be solved for antigene treatments to succeed (302;303). A more unique problem related to triplex helix forming oligonucleotide based antigene strategies is the need of the TFO to have a strong affinity for the target whilst simultaneously maintaining sequence-specificity to avoid interaction with non-target sequences within the cell (53;54;302).

The feasibility of TFO for antigene therapy *in vivo* has been illustrated in a tumour model in mice (304). Tumours were induced in Ncr nude mice and thereafter treated with injection of triplex forming oligonucleotides. Tumour size was found to be significantly reduced compared with tumours treated with a scrambled TFO, or the vehicle on their own (304). To our knowledge, at present, no antigene treatment based on TFO has been approved for *in vivo* treatment.

Use of nucleic acid stabilizing molecules to counteract the limitations of triplex helixes

As previously stated, triplex helixes are limited by i) the mandatory polypurine nucleotide target sequence, preferably without pyrimidine nucleotide interruptions, ii) acidic pH conditions to allow formation of parallel triplex helixes, iii) avoidance of longer dG nucleotide streches in antiparallel triplex helixes, due to G-quadruplexes and iv) the slow annealing kinetics of triplex helixes.

To address these limitations, a number of different nucleic acid stabilizing molecules have been tested. Some molecules have been used specifically to counteract a single limitation, whereas others, such as ENA, LNA, PNA and PMO, have been used generally to improve the stability of the triplex helixes.

LNA containing oligonucletoides stabilize triplex helixes by decreasing the dissociation rate of the triplex helixes (305-307). Oligonucleotides with alternating LNA and DNA nucleotides have been found to increase the thermal stability of the triplex helix the most without decreasing the discrimination of base mismatches (308;309). LNA modified oligonucleotides were found to increase the cellular activity of the TFO and to increase the stability of the triplex helix (306;307). Conjugation of an acridine intercalator at the 5’ end of the LNA modified TFO was found to significantly increase the biological activity of the LNA modified oligonucleotide and to stabilize the triplex helix even further by decreasing the dissociation speed of the triplex helix (306). In a study comparing ENA and LNA modified TFO sequences, oligonucleotides completely modified with ENA nucleotides were found to allow formation of triplex helixes in contrast to oligonucleotides completely modified with LNA nucleotides (140). In general, no significant differences between LNA and ENA modified TFO sequences were found due to oligonucleotides with alternation of modified nucleotides and conventional DNA nucleotides stabilizing the triplex helix the most (140).

PMO modified TFO sequences improve the thermal stability of triplex helixes by increasing the association rate constant for formation of the triplex helix (230). PMO based TFO sequences enable formation of triplex helixes at physiological monovalent cation conctration in contrast to phosphoramidate modified TFO sequences and conventional DNA TFO sequences (229). PNA

modified TFO sequences have predominantly been used as clamp oligonucleotides (the antiparallel duplex helix strand and the triplex helix forming strand of the triplex helix are linked by a spacer and both strands have been PNA modified) (202;288;310;311). The PNA clamp oligonucleotides have been further modified with lysine, pseudoisocytosine and 9-aminoacridine. The lysines were added to the oligonucleotides to increase solubility of the PNA modified TFO sequences (311), whereas pseudoisocytosine are used as substitutions for cytosine in the PNA modified TFO sequences to decrease the pH dependence of the dC and dT nucleotide based parallel triplex helix (212;288;311). Conjugation of an acridine intercalator at the 5' end of PNA modified triplex helix forming oligonucleotides significantly stabilizes PNA clamp based triplex helix formations at physiological monovalent cation conctrations (312).

The stability of triplex helix formations can also be improved by use of charged molecules such as 5-propargylamino-dU (U^P) (313), methylthiourea-linked nucleosides (DNmt) (314) and 2'-aminoethoxy-modified oligonucleotides (2'-AE) (315). All of these molecules add positive charges to the TFO sequence and increase the association rate of the triplex helix.

The molecules mentioned above allow a general improvement in the thermal stability of triplex helixes. By combining molecules that increase the annealing rate and inhibit the dissociation rate, it may be possible to counteract the slow kinetics for formation of triplex helixes. When using molecules that inhibit the dissociation rate, it is important to recall that nucleobase mismatches increase the dissociation rate but are unaffected by the annealing rate of the triplex helix (54). It will therefore be beneficial if molecules used to inhibit the dissociation rate of the parallel triplex helix diminish the dissociation rate in a part of the oligonucleotide but not the whole oligonucleotide. Another limitation of the kinetics of dC and dT nucleotide based parallel triplex helixes is the need for protonated dC nucleotides in the TFO sequence. The studies of PNA modified triplex forming nucleotide clamps have demonstrated the feasibility of pseudoisocytosines for substitution of dC nucleotides to counteract the pH dependence of dC and dT nucleotide based parallel triplex helixes. If dC and dT nucleotide parallel triplex helixes are omitted, it will be necessary to inhibit the aggregation of TFO sequences entailing stretches of dG nucleotides into G-quadruplexes. This may partly be possible by substitution of guanine nucleobases by pyrazolo[3,4-*d*]pyrimidine guanine bases (316).

Allowing pyrimidine nucleotides in the target nucleotide sequence would make possible formation of triplex helixes with any DNA nucleotide target sequence. Pyrimidines in the target nucleotide sequence are less destabilizing when a dG nucleotide is placed in the triplex helix forming oligonucleotide (53) and can also be surpassed by the use of an abasic linker in the TFO (317). Such approaches may allow single pyrimidine nucleotides in the target nucleotide sequence, however, they will not allow specific recognition of multiple pyrimidine nucleotides in the target nucleotide sequence. To allow multiple pyrimidine nucleotides in the target sequence, it will be necessary to use molecules such as those mentioned in section 2.2.5.

Potential use of TINA molecules in clinical diagnostic assays and therapy

The ultimate goal within the field of clinical diagnostics will be to use TINA modified oligonucleotides for point-of-care diagnostics. To attain this, we first need to establish which applications TINA modified oligonucleotides add a competitive edge.

Antiparallel duplex helix based applications

The TINA molecules were developed to improve the thermal stability of triplex helixes, whereas they initially were found to decrease the thermal stability of antiparallel duplex helixes (113). Nothing indicates that TINA modified oligonucleotides are suitable for hybridization with RNA nucleotide target oligonucleotides, whereas TINA molecules stabilize hybridization with DNA target oligonucleotides, particularly if the TINA molecules are placed at the 5' and 3' ends of the oligonucleotide.

A possible application of TINA modified oligonucleotides could be as 3' modifications of qPCR probes but most likely probes conjugated to MGB or ZNA will further stabilize the qPCR probe.

At present, we have established that conjugation of a TINA molecule at the 5' end of end-point PCR primers and qPCR primers increases the efficiency of the PCR reaction significantly and allows substantial reductions in the primer concentrations without interference with mismatch discrimination of the PCR primers (unpublished data). The TINA modified primers are especially useful in multiplex PCR reactions in which it will be advantageous to decrease the primer concentrations or as a mean to specifically increase the PCR efficiency of single PCR reactions within a multiplex PCR assay (unpublished data). This ability of TINA modified primers to increase the PCR robustness is in contrast to LNA, ENA, PNA and ZNA modified primers.

Another PCR based application of TINA modified oligonucleotides is in Cliffhanger® PCR. In Cliffhanger® PCR the TINA molecule is placed in a bifunctional oligonucleotide. The part of the oligonucleotide 3' to the TINA molecule functions as a conventional PCR primer, whereas the part of the oligonucleotide 5' to the TINA molecule is a nonsense oligonucleotide tail (patent pending). This approach has multiple advantages that can be summarized in i) increased multiplex PCR efficiency, ii) improved analytical sensitivity upon detection of multiplex PCR reactions, iii) decreased nonspecific cross-reactivity in microarray and biosensor approaches for detection of multiplex PCR products and iv) easier design and more uniform PCR reactions. The TINA molecule in the bifunctional oligonucleotide still improves the efficiency of the PCR reaction, but the TINA molecule also eliminates read-through by the PCR polymerase, leaving the nonsense oligonucleotide sequence as a single stranded overhang. The single stranded PCR overhangs allow detection of the multiplex PCR reaction without preceding denaturation of PCR products thus increasing the analytical sensitivity of PCR amplicons' detection. The risk of cross-reactivity between multiple PCR amplicons is diminished because the nonsense oligonucleotide tag-sequence can be designed freely. As the PCR amplicons are discriminated by the nonsense oligonucleotide tag-sequence, the PCR amplicons can be designed with similar lengths. By equalling the lengths of the PCR amplicons, uniformized multiplex PCR efficiency can be achieved thus simplifying the optimization of the multiplex PCR assay. A final benefit of the Cliffhanger® PCR is a greater inter-assay uniformity of the multiplex PCR assay. Linkers can be used between a PCR primer and a nonsense oligonucleotide sequence, however, the nonsense oligonucleotide can flip-back onto the target nucleotide sequence and interfere with the PCR polymerase. The linkers can also coil-up and bring the nonsense oligonucleotide sequence, and the primer sequence, within close proximity allowing the PCR polymerase to read-through the linker. Such interference of the linker subsequently decreases the inter-assay uniformity of the multiplex PCR assay (manuscript in preparation).

As demonstrated, we not only try to use the TINA molecules in well-established methods for clinical diagnostics, but also to develop new methods that are based on the strengths of the TINA molecules. Another new method in development is the Cut-n-Hyb™ LCR method for detection of differential methylation (patent pending). Feasibility studies with this new method are to be started mid-April 2011.

Triplex helix based applications

As mentioned in section 4.6.3, acridine modification of PNA and LNA containing triplex helix forming oligonucleotides has been shown to increase the thermal stability of the triplex helix significantly (306;312). TINA molecules containing the pyrene moiety improve the stability of parallel triplex helix compared to TINA molecules modified by derivatives of acridine (240). The *para*-TINA molecules have been shown to increase the Tm of dC and dT nucleotide based parallel triplex helixes significantly and to allow dC and dT nucleotide based parallel triplex helixes even at pH 7.2 (113).

Even though the *para*-TINA molecule on its own stabilizes triplex helixes, it will be advantageous to combine the *para*-TINA molecule with positively charged molecules that increase the association rate of the triplex helix, such as 2'-AE-modified T nucleotides (315). This combination will hopefully improve the kinetic for formation of the triplex helix significantly. Through additional substitution of dC by a molecule that removes the pH dependence of dC and dT nucleotide based parallel triplex helixes, such as 2'-*O*-methylpseudoisocytidine (194), it is likely to stabilize the dC and dT nucleotide based parallel triplex helixes even more. Another way to improve the stability of the dC and dT nucleotide based parallel triplex helixes will be to use oligonucleotide clamps consisting of the antiparallel duplex helix strand and the parallel triplex helix forming oligonucleotide linked together by a spacer instead of a triplex helix forming oligonucleotide on its own (unpublished data). Oligonucleotide clamps will not only improve the thermal stability of the triplex helixes, it will also ensure the utmost discrimination of nucleobase mismatches in the target oligonucleotide strand because the nucleobase mismatch will impact the stability of both the triplex helix forming oligonucleotide strand and the antiparallel duplex forming strand of the triplex helixes (51;52).

We have demonstrated in MII that TINA molecules are beneficial when placed at the 5' and 3' ends of the dC and dT nucleotide based parallel triplex helixes at acidic pH, whereas they were more stabilizing when placed centrally as pH increased towards neutral pH. If the pH dependence of dC and dT nucleotide based parallel triplex helixes is counteracted by substitution of dC nucleotides by pH neutralizing molecules, it is likely that the design rules for stabilizing parallel triplex helixes by TINA molecules will follow the design rules established at acidic pH for dC and dT nucleotide based parallel triplex helixes.

The described heavily modified triplex helix forming oligonucleotides will still rely on the mandatory polypurine nucleotide target sequence of triplex helixes. To allow triplex helixes with target nucleotide sequences entailing all four nucleobases in DNA, it will be necessary to modify the TFO sequence even further. Doing so, the molecules that allow pyrimidines in the target nucleotide sequence should be chosen to allow specific discrimination between dC and dT nucleobases in the target nucleotide sequence. This may be achieved by use of ^{A}PP and S nucleobases to counteract the dC and dT nucleotides in the target nucleotide sequence (175).

The use of triplex helix forming oligonucleotides clamps modified by *para*-TINA, a molecule for substitution of dC nucleotides and a positively charged molecule that increase the association rate of triplex helixes, will hopefully allow improvement of assays for purification and isolation of intact dsDNA at neutral pH. Another application of such a combination of molecules would be to improve the mismatch discrimination and analytical sensitivity of triplex helix based biosensors.

Para-TINA modified oligonucleotides are still to be exploited for *in vitro* cellular systems and for *in vivo* therapy. *Para*-TINA modified oligonucleotides may be useful as triplex helix forming oligonucleotides for antigene therapy and as G-quadruplex forming oligonucleotides that bind to protein targets in a shape-specific recognition in aptamer therapy (233;301;302). To allow *in vivo* therapy by *para*-TINA modified oligonucleotides, issues regarding delivery, stability, affinity and sequence-specificity need to be addressed. The structure of TINA molecules differs significantly from DNA nucleic acids and is resistant to nucleases in *in vitro* assays (unpublished data). The annealing kinetic of *para*-TINA modified dC and dT nucleotide based parallel triplex helix forming oligonucleotides *in vivo* is likely to be slow. This may not be a problem as long as the stability toward intracellular nucleases is sufficient, but it is tempting to combine *para*-TINA molecules with other molecules to improve the stability of the dC and dT nucleotide based parallel triplex helixes. Such heavily modified oligonucleotides may increase the affinity for the intracellular triplex helix forming target but may also induce nonspecific binding to mismatched nucleotide sequences (318). The combination of different triplex helix stabilizing molecules for *in vivo* therapy must therefore be considered carefully. The structure of the *para*-TINA molecule may potentially enhance mutagenesis and carcinogenesis *in vivo*. This is a concern since aromatic structures, such as pyrenes, have been used to break DNA strands and induce mutations when conjugated to TFO sequences (292). Finally, the metabolization and secretion of degraded *para*-TINA modified oligonucleotides *in vivo* needs to be clarified. It has previously been speculated that released modified oligonucleotides from antisense oligonucleotides may be reincorporated into and mutagenize cellular DNA and, likewise, it will be necessary to evaluate the biosafety of *para*-TINA modified oligonucleotides for *in vivo* therapy (303).

CONCLUSIONS

The design rules for placement of TINA molecules in antiparallel duplex helixes and in dC and dT nucleotide based parallel triplex helixes are simple and robust. In antiparallel duplex helixes, an *ortho*-TINA molecule should be placed terminally at each end of the oligonucleotide. The analytical sensitivity for capture of denatured PCR amplicons by *ortho*-TINA modified oligonucleotides is significantly improved. The improvement in analytical sensitivity

by *ortho*-TINA modified oligonucleotides is particularly evident at stringent assay conditions.

For thermal stabilization of parallel triplex helixes, the *para*-TINA molecule is preferable. At pH below 6.0, *para*-TINA molecules are optimally positioned terminally with additional *para*-

TINA molecules placed for each half or whole helix turn in the triplex helix. At pH above 6.0, *para*-TINA molecules should be placed in the centre of the triplex helix forming oligonucleotide with a *para*-TINA molecule for each half or whole helix turn in the triplex helix. Two *para*-TINA molecules should always be placed with at least three to four nucleotides in-between. When intercalating directly adjacent to a nucleobase mismatch, a *para*-TINA

molecule minimizes the Tm decrease caused by the nucleobase mismatch. Moving the *para*-TINA molecule two nucleotides away from the nucleobase mismatch eliminates the decreased nucleobase mismatch discrimination. Design rules for placement of *para*-TINA molecules in antiparallel triplex helixes are yet to be determined.

Combining TINA modified triplex forming clamp oligonucleotides with molecules that improve the annealing rate for formation of parallel triplex helixes as well as those that counteract the pH dependence of dC and dT nucleotide based parallel triplex helixes may be beneficial to the development of diagnostic assays based on parallel triplex helixes. Such combinations are likely to improve present assays for purification and isolation of dsDNA as well as detection of DNA targets using biosensors with increased nucleobase mismatch discrimination due to the triplex forming clamp oligonucleotides.

The *ortho*-TINA molecule is suited for improving the stability of diagnostic assays based on antiparallel duplex helixes. The efficiency of multiplex end-point PCR and qPCR assays, in particular, can be improved by conjugation of an *ortho*-TINA molecule to the 5’ end of the PCR primers. The TINA molecules also enable novel technologies such as Cliffhanger® PCR technology and Cut-n-Hyb™ LCR technology, which are currently in development.

ABBREVIATIONS

ΔTm	Change in Tm caused by a nucleobase mismatch
2’-AE	2’-aminoethoxy-modified oligonucleotide
AD	Antiparallel duplex helix
AD-strand	Antiparallel duplex forming strand of the triplex helix
AMANY	(*S*)-4-(4-(1*H*-Phenanthro[9,10-d]imidazol-2-yl) phenoxy)butane-1,2-dio
ANA	Altritol nucleic acid
APD	Avalanche photodiode
^{A}PP	3*H*-Pyrrolo[2,3-*d*]pyrimidin-2(7*H*)-one nucleobase
bDNA	branched DNA
BNA	Bridged Nucleic Acid (or LNA)
CeNA	Cyclohexene nucleic acid
D_3	1-(2-deoxy-β-D-ribofuranosyl)-4-(3-benzamido-phenyl)imidazole
dA	2’-deoxyribosyladenine-5’-monophosphate
dC	2’-deoxyribosylcytosine-5’-monophosphate
dG	2’-deoxyribosylguanine-5’-monophosphate
DNA	Deoxyribonucleic acid
DNmt	methylthiourea-linked nucleosides
dsDNA	Double stranded DNA
dT	2’-deoxyribosylthymine-5’-monophosphate
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ENA	2’-*O*, 4’-*C*-Ethylene-Bridged Nucleic Acid
FISH	fluorescence *in situ* hybridization
FRET	Fluorescence resonance energy transfer
HEG spacer	Hexaethyleneglycol spacer
HNA	Hexitol nucleic acid
INA	Intercalating Nucleic Acid ((*R*)-1-*O*-(1-pyrenyl-methyl)glycerol)
LNA	Locked Nucleic Acid (or BNA)
MagPlex™	Paramagnetic carboxyl-group coated Microspheres
MFI	Median Fluorescence Intensity
MGB	Minor Groove Binder
MOE	2’-*O*-methoxy-ethyl RNA
Naphthalimide-TINA	(*R*)-6-{[4-({ [2,3-Dihydroxypropyl]oxy}-methyl)phenyl]ethynyl}-2-[2-(dimethylamino)-ethyl]-1H-benzo[de]isoquinoline-1,3(2H)-dione)
Naphthalene-TINA	(*R*)-1-*O*-[4-(1-pyrenylethynyl)naphtalen-1-ylmethyl]glycerol
Ortho-TINA	(*R*)-1-*O*-[2-(1-pyrenylethynyl)phenylmethyl]-glycerol
Para-TINA	(*R*)-1-*O*-[4-(1-pyrenylethynyl)phenylmethyl]-glycerol
PCR	Polymerase Chain Reaction
PMO	Phosphoramidate Morpholino Oligonucleotide
PNA	Peptide Nucleic Acid
PS	Phosphorothioate DNA
PT	Parallel triplex helix
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain Reaction
S	*N*-(4-(3-acetamidophenyl)thiazol-2-yl)acetamide nucleobase
SA-PE	Streptavidin-*R*-phycoerythrin
ssDNA	Single-stranded DNA
Sulfo-NHS	*N*-hydroxysulfosuccinimide
TFO	Triplex Forming Oligonucleotide
TINA	Twisted Intercalating Nucleic Acid
Tm	Melting temperature
TMAC	Tetramethylammonium chloride
U	2’-ribosyluracil-5’-monophosphate
UNA	Unlocked Nucleic Acid
U^{P}	5-propargylamino-dU
*x*MAP	Luminex® microspheres coated with carboxyl-groups
xTAG	Luminex® microspheres coated with short oligo-nucleotide tags
ZNA	Zipped Nucleic Acid

SUMMARY

This thesis establishes oligonucleotide design rules and applications of a novel group of DNA stabilizing molecules collectively called Twisted Intercalating Nucleic Acid – TINA. Three peer-reviewed publications form the basis for the thesis. One publication describes an improved and rapid method for determination of DNA melting points and two publications describe the effects of positioning TINA molecules in parallel triplex helix and antiparallel duplex helix forming DNA structures. The third publication establishes that TINA molecules containing oligonucleotides improve an antiparallel duplex hybridization based capture assay's analytical sensitivity compared to conventionel DNA oligonucleotides.

Clinical microbiology is traditionally based on pathogenic microorganisms' culture and serological tests. The introduction of DNA target amplification methods like PCR has improved the analytical sensitivity and total turn around time involved in clinical diagnostics of infections. Due to the relatively weak hybridization between the two strands of double stranded DNA, a number of nucleic acid stabilizing molecules have been developed to improve the sensitivity of DNA based diagnostics through superior binding properties.

A short introduction is given to Watson-Crick and Hoogsteen based DNA binding and the derived DNA structures. A number of

other nucleic acid stabilizing molecules are described. The stabilizing effect of TINA molecules on different DNA structures is discussed and considered in relation to other nucleic acid stabilizing molecules and in relation to future use of TINA containing oligonucleotides in clinical diagnostics and therapy.

In conclusion, design of TINA modified oligonucleotides for antiparallel duplex helixes and parallel triplex helixes follows simple purpose dependent rules. TINA molecules are well suited for improving multiplex PCR assays and can be used as part of novel technologies. Future research should test whether combinations of TINA molecules and other nucleic acid stabilizing molecules can increase analytical sensitivity whilst maintaining nucleobase mismatch discrimination in triplex helix based diagnostic assays.

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