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# Optimal design of parallel triplex forming oligonucleotides containing Twisted Intercalating Nucleic Acids—TINA

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## ABSTRACT

**Twisted intercalating nucleic acid (TINA) is a novel intercalator and stabilizer of Hoogsteen type parallel triplex formations (PT). Specific design rules for position of TINA in triplex forming oligonucleotides (TFOs) have not previously been presented. We describe a complete collection of easy and robust design rules based upon more than 2500 melting points ($T_m$) determined by FRET. To increase the sensitivity of PT, multiple TINAs should be placed with at least 3 nt in-between or preferable one TINA for each half helixturn and/or whole helixturn. We find that $\Delta T_m$ of base mismatches on PT is remarkably high (between 7.4 and 15.2°C) compared to antiparallel duplexes (between 3.8 and 9.4°C). The specificity of PT by $\Delta T_m$ increases when shorter TFOs and higher pH are chosen. To increase $\Delta T$ms, base mismatches should be placed in the center of the TFO and when feasible, A, C or T to G base mismatches should be avoided. Base mismatches can be neutralized by intercalation of a TINA on each side of the base mismatch and masked by a TINA intercalating direct 3′ (preferable) or 5′ of it. We predict that TINA stabilized PT will improve the sensitivity and specificity of DNA based clinical diagnostic assays.**

## INTRODUCTION

Twisted intercalating nucleic acid (TINA) is a novel artificial intercalating nucleic acid, which is capable of stabilizing Hoogsteen-type DNA triplex formations (1). Currently, limited knowledge concerning design of oligonucleotides containing TINA is available.

Preceding data suggest that Hoogsteen-type DNA triplex formations are characterized by a pronounced decrease in melting point ($T_m$) per base mismatch (1,2), which is in contrast to the relatively low change in $T_m$ ($\Delta T_m$) per base mismatch seen in antiparallel duplex (AD) formations (2). Triplex formations are divided into parallel and antiparallel triplexes by the orientation of the Triplex Forming Oligonucleotide (TFO) (3). The TFOs of parallel triplexes (PT) follow the orientation of the homopurine target strand and consist of CT or GT sequences (4), whereas antiparallel triplexes follow the orientation of the homopyrimidine AD strand and consist of GT or GA sequences (5). The formation of triplexes is limited by (i) dependence on DNA homopurine stretches in the target sequence (6–8), (ii) acidic pH for PT formation due to the need of protonated cytosine in the TFO (4,9,10) and (iii) quadruplex formation of guanosine-rich TFOs in antiparallel triplexes (11).

TINA has been shown to increase $T_m$ and to decrease pH dependence of PT seemingly without compromising $\Delta T_m$ (1). TINA in the TFO should be placed as an intercalator between two bases and not as a substitution for a base, and the stabilizing effect of two TINAs in the TFO increases, when the TINAs are placed with several bases in-between (1,12–14). *In vitro* gene-expression studies of antiparallel triplex formation have shown that TINA lowers the tendency of potassium induced oligonucleotide aggregation compared with DNA TFO (15). In addition, TINA has been used to flank guanosine stretches within quadruplex oligonucleotides. This lead to an increase $T_m$ of the quadruplex and induced a strong antiproliferative effect in Panc-1 cells by blocking the *KRAS* promoter (16).

Very little is known about the stabilizing effect of TINA on DNA triplex formations. During conventional oligonucleotide synthesis, TINA is covalently inserted between two bases in the phosphate backbone, and TINA is

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believed to form a bulge in the phosphate backbone while its pyrene intercalator is inserted into the base stacking of the AD part of the triplex (17). Recently, a number of TINA analogues have been synthesized, but none of them are superior stabilizers of PT compared to TINA (1,14,18–20).

The aim of this study was to verify the previously observed high $\Delta T_m$ for DNA triplex formations and to determine the optimal design rules and stabilizing effect of TINA in PT. To do so a high-speed melting curve acquisition method has been used and validated towards a slower standard method. If TINA modified Hoogsteen-type PT have increased $\Delta T_m$ compared to conventional AD formations, this could improve the specificity of future DNA-based clinical assays.

## MATERIALS AND METHODS

### Fluorescence resonance energy transfer (FRET) system

For determinations of $\Delta T_m$, a synthetic polypurine DNA target (5′ AGG GAA AAG AAG GGA GGG ATA AC 3′) was constructed to ensure different base mutations at position 4, 7, 10, 13 and 16 (GGA, AAA, GAA, GGG and AGG) from the 5′-end of the polypurine target strand. To visualize PT formation, target oligonucleotides were labelled with ATTO495 and TFOs were labelled with ATTO590. For visualization of AD formation the ATTO590 fluorophore was placed on the non-target strand of the AD. Target oligonucleotides labelled with ATTO495 were synthesized as oligonucleotides with 5′-amino-modifier-C6 and linked to the ATTO495 NHS-ester. ATTO495 is a modification of Acridine Orange with excitation maximum at 495 nm and emission maximum at 527 nm. Oligonucleotides labelled with ATTO590 were synthesized with a 5′-amino-modifier-C6 or 3′-amino-modifier-C7 and linked to ATTO590 NHS-ester. ATTO590 is a derivative of Rhodamine dyes with excitation maximum at 594 nm and emission maximum at 624 nm. The ATTO495 and ATTO590 FRET pair was excitated at 470 nm on a LightCycler 2.0 and fluorescence emission was detected at 640 nm.

### Oligonucleotides

Unlabelled and ATTO495/ATTO590-labelled oligonucleotides were purchased from IBA GmbH (Göttingen, Germany) and DNA Technology A/S (Risskov, Denmark) on a 0.2 μmol synthesis scale with high performance liquid chromatography (HPLC) purification and quality control. A 23-nt target sequence as 5′ ATTO495-AGG GAA AAG AAG GGA GGG ATA AC 3′ (D-200) with a base mismatch at either position 4, 7, 9, 10, 11, 13 or 16 or two base mismatches at position 9 and 11 from the 5′-end were combined with a 23-nt AD sequence (AD) of 5′ GTT ATC CCT CCC TTC TTT TCC CT 3′(D-256) with zero to two TINA modifications and a TFO as 5′ ATTO590-TCC CTT TTC TTC CCT CCC T 3′ (D-284) with zero to four TINA modifications and a TFO length of 7–19 nt counted from 5′ to 3′. The TINA modification is a *(R)*-1-O-[4-(1-pyrenylethynyl)phenylmethyl]glycerol and is inserted as an internal bulge in the DNA oligonucleotides. An overview of most oligonucleotides used in this study can be found in Supplementary Tables S1, S2 and S3.

### Melting curve acquisition

Melting curve experiments were performed on a LightCycler 2.0 (Roche Applied Science, Basel, Switzerland) as previously described and validated (21,22). In short, PT experiments were performed in 20 μl LightCycler capillaries using 1.0 μM of each oligonuclotide in sodium acetate buffer (50 mM NaOAc, 100 mM NaCl and 10 mM $MgCl_2$) at pH 4.5 (experiments with shorter TFOs), pH 5.0 (standard conditions), pH 5.5 or pH 6.0 (pH validation and comparison of $\Delta T_m$ for AD and PT). AD experiments were performed with 0.5 μM of each oligonucleotide in sodium phosphate buffer (50 mM $NaH_2PO_4/Na_2HPO_4$, 100 mM NaCl and 0.1 mM EDTA) at pH 5.5 or pH 6.0 (comparison of $\Delta T_m$ for AD and PT) and pH 7.0 (standard conditions). All chemicals were purchased from Sigma-Aldrich Denmark (Brøndby, Denmark). $T_m$ measurements were carried out using a high-speed standard program of (i) a dissociation step from 37 to 95°C with a ramp rate of 0.2°C/s and hold for 15 s at 95°C, (ii) annealing from 95 to 37°C with a ramp rate of 0.2°C/s and hold for 5 min at 37°C and (iii) dissociation step from 37 to 95°C with a ramp rate of 0.05°C/s and continued measurement of fluorescence. $T_m$ was identified using the LightCycler Software 4.1 for melting curve analysis and defined as the peak of the first derivative.

All melting curve determinations were conducted as single capillary measurements. A setup control composed of D-200, D-256 and D-284 was included in all runs. Prior to $T_m$ identification, runs were colour compensated by subtraction of the fluorophore background fluorescence.

### Method validation

We have previously validated the use of ATTO fluorophores for PT and AD FRET on the LightCycler and compared it to UV absorbance measurements (22). The present high-speed LightCycler method uses a ramp rate of 3°C/min, which is too fast to allow the PT to be in thermodynamic equilibrium under dissociation. Thus, the reported $T_m$ values are overestimates. Consequently, the results were validated doing annealing curves by (i) heating to 95°C at a ramp rate of 0.2°C/s and hold for 10 min, (ii) cooling from 95 to 37°C leaving the samples to equilibrate for 5 min before each fluorescence reading, leading to one fluorescence reading per 1°C. In addition dissociation curves where done by (i) leaving the capillaries for 30 min at 37°C, (ii) heating from 37 to 95°C leaving the samples to equilibrate for 5 min before each fluorescence reading. This method is routinely used on the LightCycler to avoid significant hysteresis between the dissociation and annealing curves (23). Validation data for (i) dissociation speed (3, 1, 0.5 and 0.2°C/min), (ii) time before dissociation (5 min, 30 min, 1 h and 24 h),

(iii) $T_m$ comparisons for TINA oligonucleotides (at pH 5.0, 5.5 and 6.0) and (iv) $\Delta T_m$ comparisons for AD and PT (at pH 5.0, 5.5 and 6.0 in sodium acetate buffer (50 mM NaOAc, 100 mM NaCl and 10 mM $MgCl_2$) or phosphate buffer (50 mM $NaH_2PO_4/Na_2HPO_4$, 100 mM NaCl and 0.1 mM EDTA) at pH 5.5, 6.0 and 7.0) were done comparing annealing and dissociation curves at a ramp rate of 0.2°C/min and 3°C/min. Data are included in Supplementary Tables S5–S8.

## RESULTS

### $T_m$ determinations and design rules for parallel triplex with TINA in the TFO

Table 1 highlights the effects of TINAs inserted in the TFO. A complete list of tested TINA positions in the TFO and $T_m$ data can be found in Supplementary Table S1. On average, one TINA in the TFO increased $T_m$ by 3.9°C (range 0.6–5.9°C), whereas two TINAs in the TFO increased $T_m$ by an average of 6.5°C (range 1.9–9.9°C). As illustrated in Table 1 the number of bases in-between two TINAs had a huge impact on $T_m$. Based on all *T*ms in Supplementary Table 1 we found that two to four TINAs should be placed with (i) at least three bases in-between, (ii) an optimum of 5–7 or 10–14 bases in-between (equivalent to a half or a full helix-turn) and (iii) a TINA at each end of the oligonucleotide.

### The effect of base mismatches in the target sequence on $\Delta T_m$ of the TFO

A number of base mismatches at different positions in the target sequence were tested. Figure 1 shows the effect of the base mismatches in the target oligonucleotide on $T_m$ of the TFO. Central base mismatches at base 9–11 in the target oligonucleotide decreased $T_m$ of the TFO with 10.7–15.2°C, whereas base mismatches towards the 5′ (base 4) and 3′-end (base 16) of the target oligonucleotide decreased $T_m$ of the TFO with 7.6–8.3°C. Adenine to guanine mutations were less destabilizing than guanine to adenine base mismatches, whereas purine to either thymine or cytosine base mismatches were equally, but less destabilizing than guanine to adenine mutations. A double mutation of guanine to adenine at position 9 and adenine to guanine at position 11 in the target oligonucleotide had a $\Delta T_m$ on the TFO of 20.8°C compared with 15.2°C for the guanine to adenine base mismatch at position 9 and 10.7°C for the adenine to guanine base mismatch at position 11 in the target oligonucleotide ($T_m$ data in Supplementary Table S1).

### The effect of base mismatches in the target sequence on $\Delta T_m$ of the Watson–Crick type AD

Base mismatches in the target sequence decreased $T_m$ of the AD with 3.8–9.4°C (Figure 1). For all positions $\Delta T_m$ of a base mismatch in the PT was higher compared with a $\Delta T_m$ of the AD. Loss of A:T base pairs where less destabilizing than loss of G:C base pairs. The double mutation of guanine to adenine at position 9 and adenine to guanine at position 11 in the target oligonucleotide had a $\Delta T_m$ of 11.1°C compared with 8.3°C for the guanine to adenine base mismatch at position 9 and 4.3°C for the adenine to guanine base mismatch at position 11 in the target oligonucleotide (Supplementary Table S1).

### Neutralizing the effect of base mismatches in the target strand by TINAs in the TFO

The $\Delta T_m$ of a base mismatch in the target strand was reduced by positioning a TINA directly adjacent. The effect was particular pronounced when TINA was placed directly 3′ to the base mismatch and less pronounced at the 5′ position of the base mismatch (Table 1: D-294/D-295, D-300/D-301, D-343/D-344). When TINA was moved away from the base mismatch its effect on $\Delta T_m$ declined (Table 1: D-299/D-300). Positioning a TINA in the TFO on each side of an adenine to cytosine base mismatch in the target strand almost neutralized the $\Delta T_m$ of the base mismatch on the TFO (Table 1: D-305 and D-426).

### Changes in $T_m$ and $\Delta T_m$ of the TFO caused by TINAs in the AD strand

To evaluate whether TINAs within the AD strand stabilized the TFO, eight oligonucleotides with one TINA and six oligonucleotides with two TINAs were tested and the results are presented in Table 2 and Supplementary Table S2. On average, one TINA in the AD increased $T_m$ of the TFO with 3.9°C (range 1.5–6.5°C), whereas a TINA placed in the AD outside the triplex forming part only increased $T_m$ with 0.8°C (Table 2: D-452). Two TINAs in the AD stabilized the TFO, particularly when they were placed with at least three bases in-between and preferable with five bases in-between (Table 2: D-499/D-500). A TINA in the AD strand 3′ to a base mismatch in the target strand lowered $\Delta T_m$ of the base mismatch (Table 2: D-448 and D-450). Two TINAs in the AD flanking an adenine to cytosine base mismatch in the target strand partially concealed the effect of the base mismatch on TFO $\Delta T_m$ (Table 2: D-498). The best way to mask the base mismatch is to place the TINA in the TFO rather than in the AD strand (Table 2: D-498/Table 1: D-305, Table 2: D-448/Table 1: D-294, Table 2: D-450/Table 1: D-300).

### Triplexes with TINA in both the TFO and AD strands

To evaluate triplex stability with TINA in both the AD and TFO strand, we combined oligonucleotides with two TINAs in both the AD and TFO strands. The effect on $T_m$ was compared with $T_m$ of the pure DNA triplex and the changes in $T_m$ are plotted in Figure 2 ($T_m$ data in Supplementary Table S3). As previously seen, $T_m$ increased as the two TINAs in each strand were moved apart and $T_m$ increased up to 12.5°C (Figure 2: red area). As previously observed, positioning of two TINAs with less than three bases in-between only slightly increased $T_m$ (Figure 2: green stretches). All positions of two TINAs directly opposite each other in the AD and TFO strands decreased the stability of the triplex (Figure 2: blue and green spots).

**Table 1.** TINA in triplex TFO and change in $T_m$ and $\Delta T_m$

| Target / TFO | **Central mismatch or 3′ mismatch in Target** Constant AD: 3′ TCC CTT TTC TTC CCT CCC TAT TG 5′ (D-256) | Match: 5′ ATTO495-AGG GAA AAG AAG GGA GGG ATA AC 3′ (D-200) | TINA effect on $T_m$ | Mismatch: 5′ ATTO495-AGG GAA AAG CAG GGA GGG ATA AC 3′ (D-213) | $\Delta T_m$ | Mismatch: 5′ ATTO495-AGG GAA AAG AAG GGA CGG ATA AC 3′ (D-210) | $\Delta T_m$ |
|---|---|---|---|---|---|---|---|
| LC D-284 | 5′ ATTO590- TCC CTT TTC TTC CCT CCC T 3′ | 73.5 | | 60.9 | -12.6 | 65.8 | -7.8 |
| LC D-293 | ATTO590- TCC CTT TT**X**C TTC CCT CCC T | 78.9 | 5.4 | 67.1 | -11.8 | | |
| LC D-294 | ATTO590- TCC CTT TTC**X** TTC CCT CCC T | 78.3 | 4.8 | 70.7 | **-7.6** | | |
| LC D-295 | ATTO590- TCC CTT TTC T**X**TC CCT CCC T | 79.3 | 5.8 | 72.6 | **-6.7** | | |
| LC D-297 | ATTO590- TCC CTT TTC TTC**X** CCT CCC T | 78.3 | 4.8 | 64.6 | -13.7 | | |
| LC D-299 | ATTO590- TCC CTT TTC TTC CC**X**T CCC T | 78.2 | 4.6 | | | 71.2 | -7.0 |
| LC D-300 | ATTO590- TCC CTT TTC TTC CCT**X** CCC T | 79.1 | 5.6 | | | 75.8 | **-3.3** |
| LC D-301 | ATTO590- TCC CTT TTC TTC CCT C**X**CC T | 77.0 | 3.5 | | | 74.4 | **-2.6** |
| LC D-304 | ATTO590- TCC CTT TTC TTC CCT CCC T**X** | 78.4 | 4.8 | | | 70.7 | -7.6 |
| LC D-305 | ATTO590- TCC CTT TTC**X** T**X**TC CCT CCC T | 75.7 | 2.1 | 75.0 | **-0.7** | | |
| LC D-307 | ATTO590- TCC CTT TT**X**C TT**X**C CCT CCC T | 80.2 | 6.7 | 72.2 | -8.0 | | |
| LC D-309A | ATTO590- TCC CTT T**X**TC TTC**X** CCT CCC T | 81.1 | 7.6 | 69.8 | -11.3 | | |
| LC D-310 | ATTO590- TCC CTT**X** TTC TTC C**X**CT CCC T | 80.3 | 6.8 | 68.3 | -12.0 | | |
| LC D-313 | ATTO590- TCC CT**X**T TTC TTC CCT**X** CCC T | 82.0 | 8.5 | 70.1 | -11.9 | | |
| LC D-231 | ATTO590- TCC**X** CTT TTC TTC CCT CC**X**C T | 79.5 | 6.0 | 67.0 | -12.5 | | |
| LC D-236 | ATTO590- **X**TCC CTT TTC TTC CCT CCC T**X** | 81.0 | 7.4 | 67.6 | -13.3 | 73.3 | -7.6 |
| LC D-341 | ATTO590- TCC CTT TTC TTC CCT CCC**X** T**X** | 77.2 | 3.7 | | | 71.4 | -5.9 |
| LC D-343 | ATTO590- TCC CTT TTC TTC CCT C**X**CC T**X** | 80.5 | 6.9 | | | 77.8 | **-2.7** |
| LC D-344 | ATTO590- TCC CTT TTC TTC CCT**X** CCC T**X** | 82.1 | 8.6 | | | 78.3 | **-3.8** |
| LC D-346 | ATTO590- TCC CTT TTC TTC C**X**CT CCC T**X** | 82.6 | 9.1 | | | 73.9 | -8.8 |
| LC D-347 | ATTO590- TCC CTT TTC TTC**X** CCT CCC T**X** | 82.0 | 8.4 | | | 73.7 | -8.3 |
| LC D-237 | ATTO590- TCC CTT TT**X**C TTC CCT CCC T**X** | 82.3 | 8.8 | | | 76.2 | -6.1 |
| LC D-426 | ATTO590- TCC CTT TTC**X** T**X**T**X**C CCT CCC T | 73.3 | -0.2 | 71.8 | **-1.5** | | |
| LC D-428 | ATTO590- TCC CTT TT**X**C T**X**TC **X**CCT CCC T | 80.4 | 6.9 | 73.7 | **-6.7** | | |
| LC D-254 | ATTO590- TCC CTT TT**X**C TTC**X** CCT C**X**CC T | 83.1 | 9.6 | 71.7 | -11.4 | | |
| LC D-435 | ATTO590- TCC CTT T**X**TC TTC**X** CCT CCC**X** T | 84.6 | 11.1 | 73.0 | -11.7 | | |
| LC D-584 | ATTO590- **X**TCC CTT TTC T**X**TC CCT CCC T**X** | 83.5 | 10.0 | 75.4 | **-8.1** | 77.6 | -5.9 |
| LC D-438 | ATTO590- **X**TCC CTT**X** TTC T**X**C**X** CCT CCC **X**T | 86.8 | 13.3 | 74.9 | -11.9 | 81.7 | -5.1 |

Change in triplex $T_m$ by introduction of one to four TINAs in the TFO and change in $\Delta T_m$ by introduction of a central base mismatch or a base mismatch towards the 3′ end of the target strand. $T_m$ was determined using 1.0 μM of each strand in NaOAc-buffer at pH 5.0. X is TINA. Base mismatches are underlined and marked in bold blue.

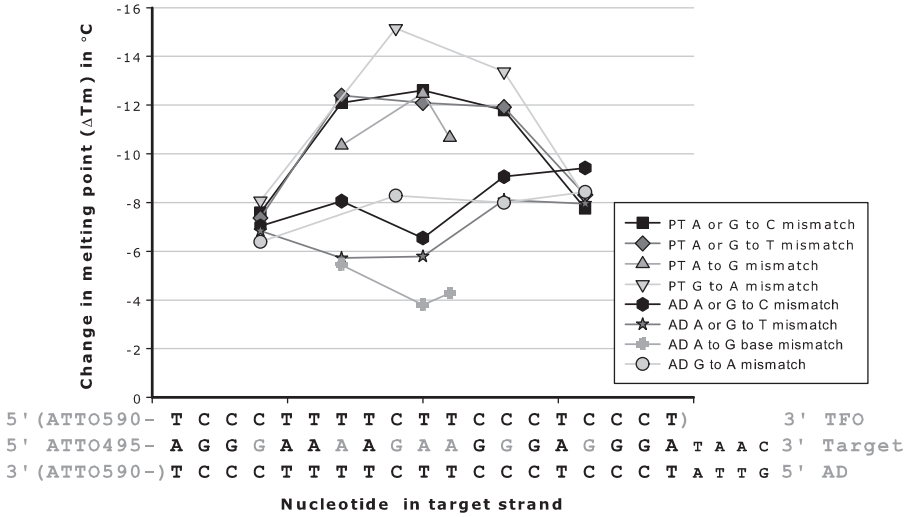



**Figure 1.** Introduction of a base mismatch along the target strand and change in melting point ($\Delta T_m$) of the AD and the parallel triplex TFO. The ADs consist of the oligonucleotides D-200 to D-222 as target strands with Base mismatches in combination with D-484 as AD strand. The PT consist of the oligonucleotides D-200 to D-222 as target strands with base mismatches in combination with D-256 as AD strand and D-284 as triplex TFO strand. Triplex melting points were determined using 1.0 μM of each strand in sodium acetate buffer at pH 5.0. AD melting points were determined using 0.5 μM of each strand in sodium phosphate buffer at pH 7.0. Base mismatch positions are marked in gray. Fluorophore and TFO strand are placed in brackets as they are not used in all experiments. PT is parallel triplex, AD is antiparallel duplex, A is adenine, G is guanine, C is cytosine and T is thymine.

### Changes in $T_m$ and $\Delta T_m$ by the length and number of TINAs in the TFO

To confirm that the positive effect on $T_m$ of TINA in the TFO was not restricted to a TFO length of 19 nt at pH 5.0, we tried a number of shorter TFOs at pH 4.5. Experiments were conducted at pH 4.5 to ensure the outmost stability of the DNA triplexes and are presented in Figures 3 and 4. Shorter TFOs decreased $T_m$ of the TFOs as seen in Figure 3. The effect of TINA on $T_m$ was enhanced as the TFO were shortened, e.g. three TINAs in the 19 oligonucleotide TFO increased $T_m$ by 4.5°C, whereas three TINAs in the 10-nt TFO increased $T_m$ by 18.4°C compared with the DNA TFO (Figure 3). At a TFO length below 9 nt, the $T_m$ of TFOs with two TINAs was higher than $T_m$ of TFOs with three TINAs, whereas TFOs longer than 9 nt were stabilized by a third TINA in the center of the TFO. Shorter TFOs increased $\Delta T_m$ of a cytosine to guanine base mismatch at position 4 in the target oligonucleotide (Figure 4). An identical increase in $\Delta T_m$ was found for DNA TFOs and TFOs with three TINAs. When the TFO length was reduced to 7–8 nt the TINA in the middle of the TFO was placed adjacent at the 3′ position of the base mismatch in the target, which lead to a stabilization and thereby decrease in $\Delta T_m$ of the TFO as illustrated in Figure 4 and previously demonstrated in Table 1 for TFOs with TINA.

### Influence of LightCycler 2.0 program on $T_m$ of PT

Since PT are not in thermodynamic equilibrium at a dissociation ramp rate of 3°C/min, we evaluated the PT shown in Table 1 at slower dissociation and annealing rates (3, 1, 0.5 and 0.2°C/min) to demonstrate that slower ramp rates leads to a parallel shift in $T_m$. Lowering the ramp rate from 3°C/min to 0.2°C/min for the TINA PT caused a parallel shifted decrease in dissociation $T_m$ of 5.3°C (range 4.6–5.7°C) (Supplementary Table S5). It was also tested, whether an incubation time of 5 min before dissociation experiments was sufficient to form stable PT. To evaluate this we preincubated the PT shown in Table 1 for 5 min, 30 min, 1 h or 24 h before dissociation experiments. Preincubation time did not influence the *T*ms determinated at a ramp rate of 3°C/min or 0.2°C/min (Supplementary Table S6).

### Changes in $T_m$ of PT with TINA at pH above 5.0

To evaluate how increasing pH influenced the $T_m$ determination of PT with TINA we increased pH from 5.0 to 5.5 and 6.0 for the PT shown in Table 1 at a ramp rate of 3°C/min and 0.2°C/min. The stabilizing effect of TINA on $T_m$ increased by increasing pH and was independent of ramp rate. Increasing pH did favour multiple TINAs and internal placement of TINAs with a half or whole helixturn in-between compared to placement of TINA only at the ends of the TFO (Supplementary Table S7).

### Comparison of parallel triplex and AD $\Delta T_m$ in identical buffer at pH 5.0 and 5.5

In Figure 1 we compared $\Delta T_m$ of PT in sodium acetate buffer at pH 5.0 with 10 mM magnesium chloride and AD in phosphate buffer at pH 7.0 without magnesium chloride. This was done to ensure maximum stability of PT and stringent hybridization conditions for $\Delta T_m$ of AD. To compare $\Delta T_m$ of AD and PT at equal pH, we changed the AD buffer to sodium acetate buffer and compared AD and PT at pH 5.0 and 5.5. The introduction of magnesium chloride increased $T_m$ of AD by approximately 4°C, but decreased $\Delta T_m$ by approximately 0.5–1°C. For PT $T_m$ decreased by approximately 14°C, whereas $\Delta T_m$ was

**Table 2.** TINA in triplex AD strand and change in $T_m$ and $\Delta T_m$

| Target / AD | **Central and 3′ mismatch in Target** Constant TFO: 5′ ATTO590-TCCCTTTTCTTCCCTCCCT 3′ (D-284) | Match 5′ ATTO495-AGGGAAAAGAAGGGAGGGA TAAC 3′ (D-200) | TINA effect on $T_m$ | Mismatch 5′ ATTO495-AGGGAAAAGCAGGGAGGGAT AAC 3′ (D-213) | $\Delta T_m$ | Mismatch 5′ ATTO495-AGGGAAAAGAAGGGACGGAT AAC 3′ (D-210) | $\Delta T_m$ |
|---|---|---|---|---|---|---|---|
| D-256 | 5′ GTT ATC CCT CCC TTC TTT TCC CT 3′ | 73.5 | | 60.9 | −12.6 | 65.8 | −7.8 |
| D-445 | GTT ATC CCT CCC TTC TTT TCC CTX | 77.1 | 3.6 | 67.0 | −10.1 | 70.7 | −6.4 |
| D-446 | GTT ATC CCT CCC TTC TTT TCXC CT | 75.0 | 1.5 | 64.1 | −10.9 | 68.3 | −6.7 |
| D-447 | GTT ATC CCT CCC TTC TTXT TCC CT | 77.6 | 4.1 | 66.1 | −11.5 | 71.7 | −6.0 |
| D-448 | GTT ATC CCT CCC TTXC TTT TCC CT | 76.9 | 3.3 | 68.8 | **−8.1** | 70.0 | −6.8 |
| D-449 | GTT ATC CCT CCXC TTC TTT TCC CT | 78.6 | 5.1 | 65.9 | −12.7 | 71.3 | −7.3 |
| D-450 | GTT ATC CCXT CCC TTC TTT TCC CT | 80.0 | 6.5 | 68.0 | −12.0 | 74.4 | **−5.6** |
| D-451 | GTT ATXC CCT CCC TTC TTT TCC CT | 77.0 | 3.5 | 64.3 | −12.7 | 69.2 | −7.8 |
| D-452 | GTXT ATC CCT CCC TTC TTT TCC CT | 74.3 | 0.8 | 61.0 | −13.3 | 67.6 | −6.7 |
| D-498 | GTT ATC CCT CCC TXTXC TTT TCC CT | 75.3 | 1.8 | 70.2 | **−5.1** | 69.1 | −6.2 |
| D-499 | GTT ATC CCT CCCX TTCX TTT TCC CT | 78.5 | 5.0 | 70.6 | −7.9 | 73.4 | −5.2 |
| D-500 | GTT ATC CCT CCXC TTC TXTT TCC CT | 80.2 | 6.7 | 69.1 | −11.1 | 74.3 | −5.9 |
| D-501 | GTT ATC CCT CXCC TTC TTXT TCC CT | 79.7 | 6.1 | 69.2 | −10.4 | 73.5 | −6.1 |
| D-502 | GTT ATC CCTX CCC TTC TTTX TCC CT | 80.6 | 7.1 | 70.6 | −10.0 | 73.7 | −6.9 |
| D-503 | GTT ATC CXCT CCC TTC TTT TCXC CT | 79.5 | 6.0 | 69.3 | −10.2 | 74.0 | **−5.6** |

Change in triplex $T_m$ by introduction of one or two TINAs in the AD strand and change in $\Delta T_m$ by introduction of a central base mismatch or a base mismatch towards the 3′ end of the target strand. $T_m$ was determined using 1.0 μM of each strand in NaOAc-buffer at pH 5.0. X is TINA. Base mismatches are underlined and marked in bold blue.

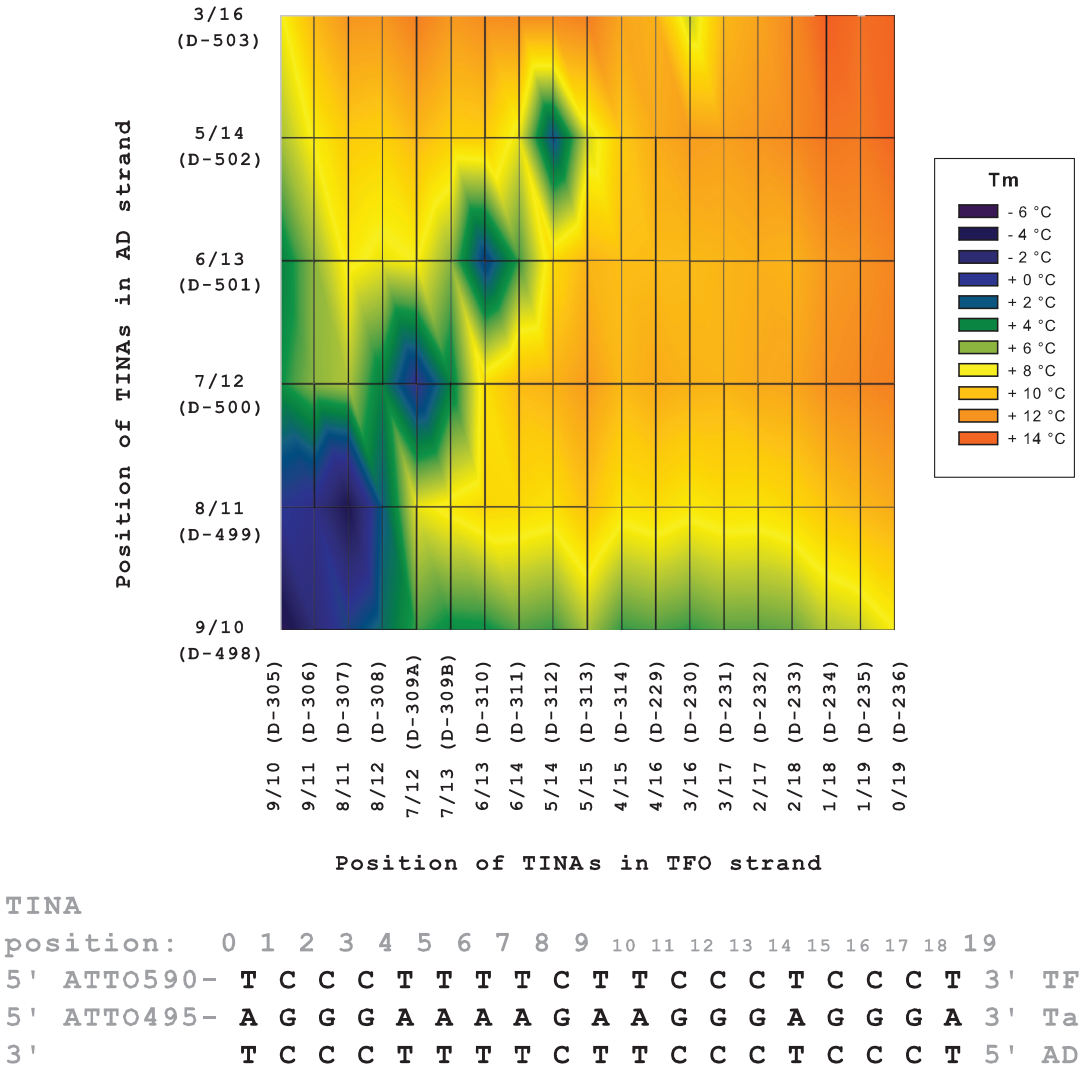



**Figure 2.** Position of two TINAs in both the TFO and AD strands of the triplex and change in $T_m$. Parallel triplex with two TINAs in the AD strand and two TINAs in the TFO strand and change in triplex $T_m$. On both axes TINAs are moved from the centre to the ends of the oligonucleotide. Triplex $T_m$ was determined using 1.0 μM of each strand in NaOAc-buffer at pH 5.0. TINA positions are indicated above the triplex sequence counted from the 5′ to the 3′ position in the target strand, and the numbers reflect the position of the base 5-prime to the TINA bulge insertion. Colour indicates the change in $T_m$ compared with a pure DNA triplex.

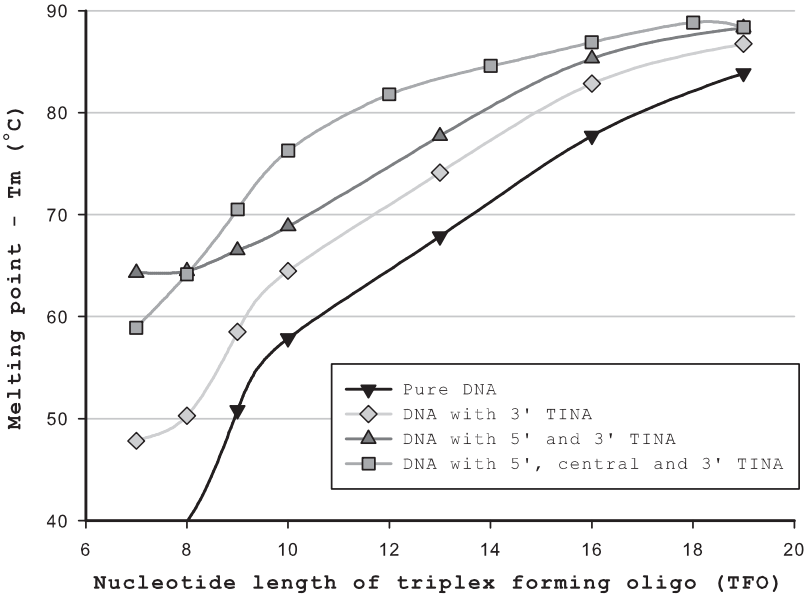
unaffected with a tendency towards a minor increase (Supplementary Table S8).

## DISCUSSION

The $\Delta T_m$ caused by a base mismatch is affected by buffer composition, the length and the sequence of the oligonucleotide and the nature and position of the base mismatch. We found that $\Delta T_m$ of base mismatches in the central part of a 23 bp AD at pH 7.0 in phosphate buffer varied between 3.8 and 9.4°C compared with 10.7–15.2°C for a central base mismatch in a 19 nt PT at pH 5.0 in sodium acetate buffer with magnesium chloride. Thus, $\Delta T_m$ of PT in the current study is remarkably high, and higher than $\Delta T_m$ of AD for all base mismatch positions tested. Experiments in sodium acetate buffer at identical pH for AD and PT showed similar results. Furthermore, $\Delta T_m$ of PT increases for shorter TFOs as seen in Figure 3. To our knowledge this is the first study comparing $\Delta T_m$ of AD and PT for multiple base mismatch positions in the target sequence containing both guanine and adenine nucleotides. The outstanding $\Delta T_m$ of PT makes PT interesting for design of highly specific assays within microbiology, forensics, cancer diagnostics, clinical genetics and *in vivo* gene regulation.

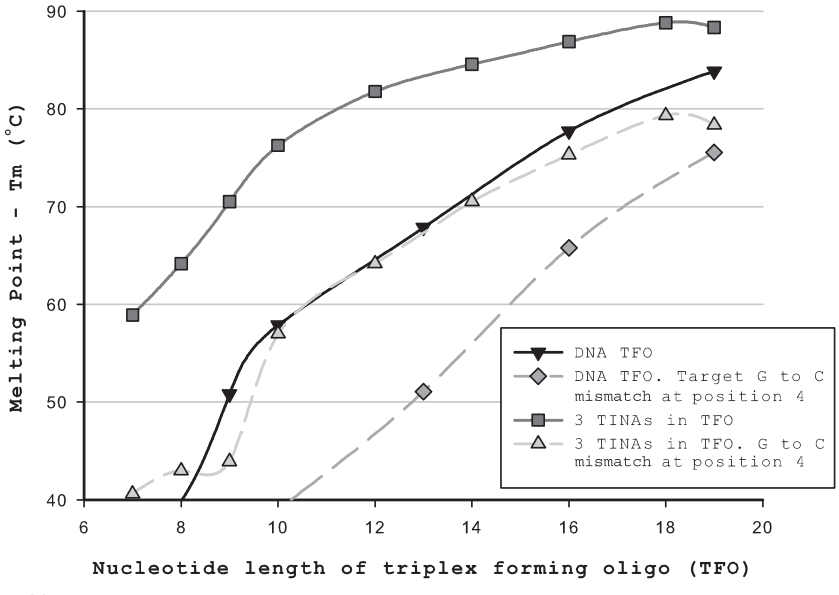
Despite the remarkable $\Delta T_m$ of PT their use is still restricted by the need of DNA homopurine stretches in the target sequence and protonation of cytosine in the TFO at acidic pH.

Since incorporation of TINA decrease the pH dependence of PT, TINAs are an obvious choice for stabilization of PT, especially since $\Delta T_m$ of PT in general is not changed by introduction of TINAs in the TFO. One exception from this rule is that TINA minimizes the $\Delta T_m$ of




| | | | | | | |
|---|---|---|---|---|---|---|
| TFO oligo | 461; | 464; 409; | 408; | 407; | 284; | |
| ID: (LC D-) | 459; | 462; 465; 467; | 588; | 589; | 304; | |
| | 509; | 512; 514; 585; | 586; | 587; | 236; | |
| | 579; | 513; 515; 470; | 580; | 581; | 582; | 583; 584. |

**Figure 3.** Effect of TFO length and number of TINAs in the TFO on triplex $T_m$. The triplexes consisted of D-200 as target strand with D-256 as AD strand and different TFO strands. The length of the TFO is counted from the 5′ to the 3′ position, and oligo IDs for TFOs used in the experiments are given below the oligo length. Oligosequences corresponding to the oligo IDs are found in Supplementary Table S4. $T_m$ was determined using 1.0 μM of each strand in sodium acetate buffer at pH 4.5.




| | | | | | | |
|---|---|---|---|---|---|---|
| TFO oligo | 461; | 464; 409; | 408; | 407; | 284; | |
| ID (LC D-): | 579; | 513; 515; 470; | 580; | 581; | 582; | 583; 584. |

**Figure 4.** $\Delta T_m$ of triplexes of different lengths with a G to C base mismatch at position 4. Length of pure DNA TFO or TFO with three TINAs and effect on $T_m$ by introduction of guanine (G) to cytosine (C) base mismatch at position 4 counted from the 5′-end of the target oligonucleotide. D-200 or D-201 as target strands in combination with D-256 as AD strand and different TFO strands. The length of the TFO is counted from the 5′ to the 3′ position, and oligo IDs for TFOs used in the experiments are given below the oligo length. Oligosequences corresponding to the oligo IDs are found in Supplementary Table S4. $T_m$ was determined using 1.0 μM of each strand in NaOAc-buffer at pH 4.5.

a base mismatch, when TINA in the TFO is placed adjacent to a base mismatch in the target strand. This is especially pronounced when TINA intercalates from the 3′ position of the mismatch base pair. This suggests that the incorporated TINAs in the TFO intercalate mostly with the base pair directly 5′ to it in the base stacking and to a lesser degree with the base pair direct 3′ to it. This masking effect has not been described previously, but

**Table 3.** Design rules for placement of TINA in PT

| |
|---|
| For maximum increase in $\Delta T_m$ for the TFO (increase sensitivity) |
| (1) Place the TINAs in the TFO and internal TINAs as a bulge (ref. no. 1) |
| (2) Place a TINA at each end of the oligonucleotide |
| (3) Always place TINAs with three or more nucleotides in-between |
| (4) Place a TINA for each half helixtum and/or whole helixtum |
| (5) For pH > 6.0 use maximum number of TINAs following rules 3 and 4 |
| (6) For oligonucleotides shorter than 9 nts use rule 2 |
| To increase $\Delta T_m$ for the TFO in parallel triplex (increase specificity) |
| (1) Position target base mismatches in the centre of the TFO |
| (2) Avoid A, C or T to G target base mismatches when feasible |
| (3) Choose a shorter TFO or higher pH to increase $\Delta T_m$ of a target base mismatch |
| (4) Place TINAs several nucleotides from the target base mismatch |
| To decrease $\Delta T_m$ for the TFO in parallel triplex (neutralize effect of base mismatch) |
| (1) Place a TINA direct 3′ of a target base mismatch to reduce $\Delta T_m$ |
| (2) Place two TINA's direct 5′ and 3′ of a target base mismatch to neutralize $\Delta T_m$ |

is especially interesting for assay development, where masking of possible base mismatches in the target sequence is of importance.

Previous publications describing the synthesis and effect of TINA in PT have indicated that two TINAs should be placed with several bases in-between to improve $T_m$ (1,12–14). In the present study we find that the optimal position of TINAs to be with 5–7 or 10–14 nt in-between, equalling a half to one helix turn of the DNA double helix. Géci *et al*. (17) have previously demonstrated that the number of pyrene intercalators in the PT decreases the pH dependent drop in $T_m$ of PT. We find that as pH increases the number of TINAs should be increased and TINAs should be included in the central part of the TFO. By optimal placement of TINAs the $T_m$, and thereby the stability of the PT, is markedly increased and will most likely enable an increase in sensitivity of clinical assays. A complete set of design rules for TINA in the TFO are presented in Table 3.

The aim of this study has been to determine the optimal design rules for placement of TINA in TFOs, and a general description regarding the thermodynamic properties of PT is beyond the scope of the present work. Hysteresis is seen for PT at the high-speed ramp rate of 3°C/min, but can as previously observed be eliminated at a ramp rate of 0.2°C/min (23,24). When we compare $T_m$s for PT at both ramp rates, we reproducibly observe a parallel shift in $T_m$. This parallel shift is equal for matching oligonucleotides and base mismatches in sodium acetate buffers with magnesium chloride at pH 5.0, 5.5 and 6.0 and for oligonucleotides with variable number of TINAs. The comparison of $\Delta T_m$ between PT and AD and between $T_m$ for oligonucleotides with different number and placement of TINAs is thereby not influenced by the faster ramp rate. We therefore believe that it is appropriate to compare $\Delta T_m$ and to give design rules for placement of TINA based on the presented data collection.

The different dissociation and annealing Tms for the TINA oligonucleotides in Table 1 are intriguing. TINA has previously been shown not only to stabilize the PT, but also to stabilize the underlying AD (1). It has been demonstrated that dissociation of PT at acidic pH can occur by (i) dissociation of the triplex strand from the PT complex and thereafter dissociation of the AD at higher temperature, (ii) a simultaneously dissociation of all three strands in the PT and (iii) in rare cases a dissociation of the AD strand of the PT followed by dissociation of the parallel duplex at higher temperature (25–30). At pH 5.0 we find that TINA modified TFOs can stabilize the whole PT leading to a dissociation $T_m$ above the AD $T_m$ meaning that the whole PT dissociates simultaneously. At higher pH the $T_m$ of the TFO with TINA drops below the $T_m$ of the underlying AD leading to dissociation of the TFO from the PT complex and thereafter dissociation at a higher temperature of the AD. Annealing of the PT is independent of pH and is based on the formation of an AD and thereafter association of the third strand to form the PT. This means that the dissociation *T*ms determined at pH 5.0 with TINAs in the TFO reflect a PT to single oligonucleotide dissociation, whereas the annealing $T_m$s reflect the formation of the AD, which is necessary for the PT formation and thereby melting curve determination in the FRET system (Supplementary Table S5).

When working with PT it is necessary to ensure that PT and not parallel duplexes are being measured. In our control measurements, $T_m$ of parallel duplex was 15.5°C lower compared to PT, which validates that the presented $T_m$s are derived from PT (Supplementary Table S1). In our study we observed a lower increase in $T_m$ when TINA was placed within the first three to four bases from the 5′-end of the TFO. This may be an effect due to the TINA, but may also be due to the fluorophores used for FRET. Incorporation of TINA may change the shape of the PT and thereby change the FRET efficiency between the ATTO495 fluorophore on the target and the ATTO590 fluorophore on the TFO. This may lead to an under-estimation of the stabilizing effect of TINA on the PT in the 5′-end of the PT.

Our determinations of the stabilizing effect of TINA in the TFO on PT are based upon a liquid phase system. We are currently developing both liquid and solid phase systems using TINA stabilized PT instead of conventional AD to increase the specificity and sensitivity of hybridization assays. Previous studies have demonstrated that DNA oligonucleotides with TINA extend the operational triplex forming pH range. Furthermore, TINA incorporation in TFOs implies reproducible positive effects on $T_m$ and $\Delta T_m$. Based on these qualities we anticipate that TINA modified oligonucleotides for triplex capture and detection, designed accordingly to the design rules for incorporation of TINA in TFOs described in this study, will be able to improve existing clinical diagnostic assay design based upon conventional Watson–Crick AD formations.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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