The use of oligodeoxynucleotide probes in chaotrope-based hybridization solutions

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ABSTRACT

Hybridization solutions containing chaotropes may be used to modulate the thermal stability (T_m or T_d) of oligodeoxynucleotide (ODN) duplexes or hybrids over a 90°C range. Modulation of T_d allows formulation of hybridization solutions that permit ambient temperature hybridization using most combinations of probe length, probe composition, target type, and facilitates development of convenient and rapid assay formats. The conditions required to achieve ODN duplex fidelity, and optimal yields of hybridized product, are described for trichloroacetate, thiocyanate, guanidinium salts and other chaotropic salts. The effects of different solid supports on T_d are described. Also, a method is presented that uses chaotropic compounds to reduce background arising from signal ODN probes in a sandwich assay hybridization format.

INTRODUCTION

Oligodeoxynucleotide probes (12–50 mers) possess some functional properties that are not shared by long DNA probes. These parameters include different rates of duplex formation as a function of a) the difference between the hybridization temperature and the T_d , b) stringency requirements for maximal selectivity/specificity of hybridization, and c) sequence-specific anomalous behavior.¹ Recently, significant advances have been made in the ability to predict ODN probe behavior in NaCl- or formamide-based hybridization solutions.^{2–4} However, there are many other hybridization solutions based on chaotropes or polar aprotic solvents that may be more advantageously employed in ODN probe-based assays. Methods for the prediction of ODN behavior in chaotrope hybridization solutions have not been previously described.

Chaotropes are particularly useful in DNA probe-based diagnostic assays, as they can simultaneously lyse the cells or organisms of interest, inhibit nucleases and proteases,^{5,6} and provide adequate hybridization stringency without chemically altering the target analyte. Chaotropic lysis and hybridization solutions eliminate the need to isolate nucleic acid prior to conducting the DNA probe assay⁷, and facilitate the development of rapid and simple assay formats. We have found that simultaneous detection of multiple different target nucleic

acids in a single sample using DNA probe technology^{8–12} is also possible. The design and use of a multi-target detection system, however, require the ability to modulate the stringency requirements for each probe used in a multi-target format such that each probe will exhibit similar properties. For ease of use, all assay steps would be ideally performed at ambient temperature, including the step of specific hybridization of target nucleic acid. In this study, we describe the effect of a series of chaotropes on the T_d , the yield of duplexes or hybrids as a function of T_d , and conditions to achieve stringency with ODN probe-based assays.

MATERIALS AND METHODS

Nomenclature

Double-strand DNA will be referred to as 'duplexes' and double strand RNA/DNA or RNA/ODN will be referred to as 'hybrids'. Double-strand oligodeoxynucleotide duplexes (12–50 mers, 'ODN/ODN') are also distinguished from long (> 100 nt) DNA duplexes ('DNA/DNA'). 'Long' RNA (> 100 nt) hybridized to ODNs will be referred to as 'RNA/ODN hybrids'. T_m refers to the temperature at which 50% of duplexes or hybrids were 'melted' or made single strand in solution as measured by spectroscopic thermal denaturation. T_d is the temperature at which 50% of one complementary strand in the duplexes or hybrids were dissociated from the 3/32nd inch diameter nylon bead solid support.¹³ The nylon bead solid support described in reference 13 has a polymer coating of poly(ethyleneimine), and oligonucleotides are covalently immobilized to the polymer (selectively) through a 5'-amine on the ODN. The polymer-coated bead derivatized with ODNs has a zwitterionic to net negative charge on the surface. This nylon bead solid support will be referred to as an 'ODN-bead' to distinguish it from nylon membranes which have entirely different surface characteristics and properties. T_d^{mem} is the temperature at which 50% of the duplexes or hybrids were dissociated from nylon membranes on which DNA or RNA is non-covalently immobilized.

In a sandwich-type assay, 'target' nucleic acid is hybridized to a 'capture' ODN probe that has been covalently immobilized on a solid support (ODN-bead). A second 'signal' nucleic acid probe (signal ODN), typically labeled with a radioisotope or a reporter group, is hybridized to a different region of the captured target nucleic acid.

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Solutions and Reagents

Filter wash (FW) is 0.09 M NaCl, 50 mM Tris pH 7.6, 25 mM EDTA. SDS/FW is FW with 0.1% sodium dodecyl sulfate (SDS). Hybridization solutions contain the text specified concentration of chaotrope [guanidinium chloride (GuCl), guanidinium thiocyanate (GuSCN), sodium thiocyanate (NaSCN), lithium trichloroacetate (LiTCA), rubidium trichloroacetate (RbTCA), sodium perchlorate (NaClO₄), potassium iodide (KI), or cesium trifluoroacetate (CsTFA)], 2% N-lauroylsarcosine (sarcosyl), 50 mM Tris pH 7.6, and 25 mM EDTA. Formamide hybridization solution contains 30% formamide, 0.09 M NaCl, 50 mM Tris-HCl pH 7.6, 5 mM EDTA and 0.1% SDS. NaCl hybridization or melting solution contained 0.18 M NaCl, 50 mM Tris-HCl pH 7.6, 5 mM EDTA and 0.1% SDS. GuSCN was purchased from Kodak (Rochester, NY). GuCl, lithium hydroxide, trichloroacetic acid, NaSCN, NaClO₄ and KI, were purchased from Sigma (St. Louis, MO). Rubidium hydroxide was purchased from CFS Chemicals (Columbus, OH). CsTFA was purchased from Pharmacia (Piscataway, NJ).

Preparation of LiTCA and RbTCA

LiTCA and RbTCA were prepared by the dropwise titration of a 3 N solution of LiOH and RbOH respectively, with trichloroacetic acid (100% w/v, 6.1 N) to pH 7.0 on ice with constant stirring. The salt was evaporated to dryness under vacuum, washed once with ether and dried.

ODN Synthesis

ODNs complementary to conserved or hypervariable regions of the 16S ribosomal RNA^{14–17} (rRNA) of *Porphyromonas gingivalis*¹⁸ (Pg), Table 1, were synthesized on either an ABI 380B or a MilliGen 7500 automated DNA synthesizer using standard cyanoethyl-N,N-diisopropylamino-phosphoramidite (CED-phosphoramidite) chemistry. Amine tails were incorporated onto the 5'-end using the commercially available N-monomethoxytritylamino-hex-6-yloxy-CED-phosphoramidite. ODNs were purified as previously described.¹³

Preparation of Nylon Bead Supports (ODN-bead)

ODN-beads (3/32nd inch diameter) were prepared as previously described.¹³ The ODN-beads contained 0.01 to 1.2 µg/bead of covalently immobilized ODN, as described in the text.

Determination of T_d and T_{opt} Values Using ODN-Beads in Various Hybridization Solutions Containing Chaotropic Salts

The method described herein for the measurement of the thermal stabilities of duplexes formed on solid supports is essentially an adaptation of methods previously described by Martinson¹⁹ for the thermal elution of DNA or RNA duplexes or hybrids from

hydroxylapatite. For the determination of ODN/ODN T_d from the ODN-bead, ³²P-labeled UP041 ODN (3'-labeled with terminal transferase²⁰) was incubated in various hybridization solutions with a complementary ODN (UP541) immobilized on ODN-beads. Five ng to 5000 ng of ³²P-ODN were hybridized in 300–400 µl volumes at ambient temperature (19–21°C) for 5–30 minutes with constant agitation. The beads were washed with 3×1 ml of the respective hybridization solution, and then once with the respective melting solution at the starting temperature of the melting process. The beads in 300–400 µl of the respective melting solution were then placed in a 0–15°C water bath. At 5 minute intervals, the temperature was raised 5°C, the solution decanted into a scintillation vial, and fresh solution (5°C below the next increment) was added to the beads. The 'melting' or duplex dissociation was conducted over a temperature range of 15°C to 80°C. Radioactivity was determined by scintillation counting.

To calculate the T_d, cumulative counts eluted at each temperature were plotted against temperature. The temperature at which 50% of the material had been dissociated from the bead was taken as the T_d. The ΔT_d (the width of the thermal melting profile) was calculated as the difference between the temperatures at which the tangent to the midpoint of the T_d curve intersected the 0 and 100% single strand axis parallels, respectively.

For the determination of RNA/ODN T_ds, rRNA was captured on ODN-beads in the presence of ³²P-labeled signal ODN (UP041) forming a capture ODN/target/signal ODN sandwich. The T_d of the individual target RNA/capture-ODN hybrid and the T_d of the target RNA/signal-ODN hybrid have been previously determined to be similar (data not shown).

For the determination of RNA/ODN or DNA/ODN T_m from nylon membranes (Schleicher & Schuell, Keene, N.H.), ³²P-labeled ODN (3'-labeled with terminal transferase²⁰) was incubated with 0.5 cm² pieces of membrane, in text-specified hybridization solutions. For the (non-covalent) immobilization of genomic DNA onto nylon membranes, purified DNA was denatured in 0.3 M NaOH at 20°C for 10 minutes. An equal volume of 2 M ammonium acetate was added and the sample was applied to Nytran membranes assembled in a slot blot apparatus. RNA was denatured in 4.6 M formaldehyde-6×SSC (0.9 M NaCl, 90 mM sodium citrate) for 15 min. at 60°C and applied to the membranes as above. After immobilization of the nucleic acids, the filters were baked at 80°C for 2 hours, then stored dry at ambient temperature. The hybridizations and dissociations were then performed as described above for the nylon bead solid supports.

To determine the T_{opt}^{ODN} (the temperature at which the maximum rate of hybridization of target nucleic acid to ODNs occurs, under near stringent to stringent conditions (–20 to –5°C below the T_d)), complementary ³²P-labeled ODN was

Table 1. ODN sequences

Name	Sequence	Function
Pg001	5'-XCAATACTCGTATCGCCCGTTATTC-3'	Pg specific capture probe
Pg006	5'-XCCTTAGGACAGTCTTCCTTCACGC-3'	
UP041	5'-XCTGCTGCCTCCCGTAGGAGT-3'	Universal signal probe
UP007	5'-XGTATTACCGCGGCTGCTG-3'	
UP003	5'-XTGACGGGCGGTGTGTACAA-3'	Complement to Pg001
Pg501	5'-XGAATAACGGGCGATACGAGTATTG-3'	
UP541	5'-XACTCCTACGGGAGGCAGCAG-3'	Complement to UP041

'X' indicates a 5'-aminohexyl tail

hybridized (to the $C_{0t_{1/2}}$) to either covalently immobilized ODN sequences on the ODN-bead as described above, or in a sandwich assay format when RNA was used as the target nucleic acid. The hybridizations were performed over a 40°C range (+ or -20°C around the T_d of the respective duplex in 5°C increments). The extent of hybridization was then measured as a function of temperature at the $C_{0t_{1/2}}$ of the respective hybridization.

Spectroscopic Thermal Denaturation

Thermal transitions determined in solution (T_m) were recorded at 260 nm using a Gilford System 2600 UV-VIS spectrophotometer equipped with a Gilford 2527 Thermo-programmer. ODNs (2 μ M/strand) were dissolved in the respective hybridization or melting solutions. The ODN mixtures were heated to 85°C, then cooled to 10–15°C to allow hybridization. The samples were slowly heated to 85°C employing a temperature increase of 0.5°C/min. Absorbance versus time was recorded, and the first derivative was computed automatically. The T_m values were determined using the first derivative maxima.

RESULTS AND DISCUSSION

Qualification of Thermal Stability (T_d) Measurements of Duplexes or Hybrids Formed on ODN-Beads

The ability to predict the thermal stability of a specifically captured target nucleic on a solid support is critical for developing ODN-probe based assays, particularly with advent of the polymerase chain reaction (PCR) technology (in which accurate and specific amplicon detection is required), or the use of ODN probes in a sandwich assay format for the direct detection of pathogen nucleic acid sequences for diagnostic purposes. Knowledge of the T_d or T_m precludes the necessity to empirically screen all possible ODN designs to achieve a specified sequence specificity at a given temperature range in a specified solution. T_d or T_m prediction is even more important when multiple ODN probes are to be used concurrently under a specified set of standard conditions to probe different target nucleic acid sequences. The general problem of coordinating probe behavior in a multiple target detection format is further complicated by the fact that T_m (the thermal transition point *in solution* where 50% of the formed duplexes or hybrids have become single strand) has not been shown to be predictive of T_d (the temperature at which 50% of a duplex or hybrid structures are dissociated from a solid support) for covalently tethered ODNs.

We demonstrate in Appendix 1 a method for the reproducible measurement of T_d values that accurately reflect or predict the thermal stability of captured target nucleic acid. The method is shown to be relatively independent of: 1) the volume of the

melting solution, 2) the concentration of ODN probe immobilized on the solid support, and 3) the length of the captured nucleic acid target.

Modulation of Thermal Melting Profiles of ODN/ODN Duplexes in Chaotrope-Based Hybridization Solutions

In traditional hybridization solutions, elevated temperatures (37–65°C) are required to achieve a high level of specificity.¹⁰ High hybridization temperatures are undesirable, however, for rapid, user-friendly diagnostic procedures. Rather, it is preferable to conduct the hybridization over a range or at a specific temperature^{21,22} near ambient temperature. To establish a high level of fidelity in an ambient temperature hybridization (18 to 25°C), the T_d of the respective duplex or hybrid should approach ambient temperature plus 5–10°C.^{23–25} Traditionally, formamide has been used to lower the T_d or T_m of an ODN duplex or hybrid,^{26–36} and urea, GuCl, salicylate, dimethyl sulfoxide and alcohols were found to potentiate the denaturation of DNA.^{37–42} We present here a systematic study of the use of chaotropic salt solutions to lower the T_d or T_m of an ODN duplex or hybrid and accomplish other goals in a comprehensive manner.

Hamaguchi and Geiduschek⁴³ first demonstrated that the thermal stability of native DNA could be varied (by as much as 60°C) in the presence of chaotropic salt solutions. Specific hybridization of long DNA probes has been shown to occur at 25–40°C in the presence of TCA, perchlorate or thiocyanate-based chaotropes.^{7,44–46} The properties of ODN probes in these conditions has not been described, however.

A comparison of a number of chaotropes and their respective properties is described in Table 2. The ability of a chaotrope to modulate the T_m or T_d of a DNA or ODN duplex is inversely proportional to the salting-out effect of the respective anionic species and directly proportional to the chaotropicity (as measured by protein dissociation) of the respective anion.^{43,46} In Figure 1, the relative effect of Cl^- , TFA^- , I^- , ClO_4^- , SCN^- and TCA^- on the thermal stability of ODN/ODN duplexes is shown. These anions span the taxigenic⁴⁷ to chaotropic range of activity. The use of chaotrope-based salt hybridization solutions allows the T_d of an ODN/ODN duplex to be modulated over a range of about 45°C (i.e., 25 to 68°C) using 3 M solutions of the respective chaotropes. The ΔT_d is about 12°C for all anions other than the trichloroacetate, which induces a sharper thermal melt curve ($\Delta T_d = 10^\circ C$). In addition, the trichloroacetate anion is the most effective counterion in lowering the T_d of ODN/ODN duplexes. In 4 M solutions of the TCA anion, the T_d of ODN/ODN duplexes (19–24 mers) is about 4°C (data not shown).

The effect of different chaotropes on the T_m or T_d of RNA/ODN hybrids is not the same as their effect on DNA/ODN duplexes. For example, the T_d of RNA/ODN hybrids and

Table 2. Relational series of chaotropic electrolyte solutions

Function	Series
Salting Out ^A	$SCN^- < ClO_4^- < NO_3^- < I^- < Br^- < Cl^- < CH_3COO^- < SO_4^- < F^-$
T_d ODN Duplex ^B	$CCl_3COO^- < GuSCN^- < NaSCN^- < ClO_4^- < I^- < CF_3COO^- < Cl^-$
T_m DNA Duplex ^C	$CCl_3COO^- < SCN^- < CF_3COO^- < ClO_4^- < I^- < CH_3COO^- < Cl^-$
Enzyme Complex Dissolution ^D	$CF_3COO^- < ClO_4^- < SCN^- < CCl_3COO^-$

^AAbility to decrease solubility of a non-electrolyte in water.⁴⁸ ^BSee Figure 1. ^CData from Hamaguchi and Geiduschek.⁴³

^DFrom Hanstein et al.⁴⁹

Figure 1. Modulation of the thermal melting profile of an ODN/ODN duplex using chaotropic hybridization solutions. ODN sequence UP541-derivatized nylon beads were hybridized with complementary ^{32}P -ODN (UP041) under stringent hybridization conditions (3 M GuSCN, 33°C). The solid supports were washed with the hybridization solution and thermally melted in 3 M solutions of RbTCA, GuSCN, NaClO₄, KI, CsTFA, or 30% formamide or 0.18 M NaCl. The % cumulative single strand material dissociated at each temperature was plotted versus temperature. The point at which 50% of the material is eluted from the bead is taken as the T_d value.

Table 3. T_d Values for ODN duplexes or hybrids in taxigenic or chaotropic hybridization solutions

Solution	Duplex Type	T_d	T_d		
			1M	2M	3M
0.18 M NaCl	ODN/ODN	68			
3.0 M NaCl	ODN/ODN	87			
CsTFA	ODN/ODN		69	67.5	64
KI	ODN/ODN		67.5	63	57.5
NaClO ₄	ODN/ODN		67.5	62	54
Formamide*	ODN/ODN	52			
GuCl	ODN/ODN		53	49	46
NaSCN	ODN/ODN		65	58	48
GuSCN	ODN/ODN		62	50	38
RbTCA	ODN/ODN		58	44	25
LiTCA	ODN/ODN		58	44	25
GuCl	RNA/ODN		52.5	49	45.5
GuSCN	RNA/ODN		51	42	37.5
LiTCA	RNA/ODN		62	51	34

*30% final concentration of formamide.

ODN/ODN duplexes differed by 9°C in 3 M LiTCA but only 0.5°C in 3 M GuSCN (Table 3). This observation suggests that it would be possible to selectively capture RNA, but not DNA targets, with immobilized ODN probes in TCA-based chaotropic hybridization solutions. This is similar to the observation of Chien and Davidson,⁴⁶ who determined that the T_{opt} (the temperature at which the maximum rate of hybridization was attained) for 23S rRNA/DNA hybrids in RbTCA was 11 degrees above the T_m for DNA/DNA duplexes.

It has been demonstrated that for any given anion, non-chaotropic counterions have little effect on the observed T_m .^{43,50} The results presented here (Table 3) confirm these observations. For example, the T_d of an ODN/ODN duplex or a RNA/ODN hybrid is identical in both LiTCA and RbTCA. However, cations can clearly have a significant effect on T_d when they exhibit chaotropic behavior, as is the case with the guanidinium cation. The T_d of an ODN/ODN duplex in 3 M GuSCN is 10°C lower than in the corresponding NaSCN solution, and the T_d in 3 M GuCl is about 40°C lower than in 3 M NaCl.

Table 4. Prediction of the T_d of ODN/ODN duplexes

Chaotrope	Function*
LiTCA	$T_d = 79 - 19 [\text{M LiTCA}]$
GuSCN	$T_d = 75 - 13 [\text{M GuSCN}]$
NaSCN	$T_d = 74 - 8.5 [\text{M NaSCN}]$
NaClO ₄	$T_d = 75 - 7 [\text{M NaClO}_4]$
KI	$T_d = 73 - 5 [\text{M KI}]$
NaCl	$T_d = 74 - 4.5 [\text{M NaCl}]$
GuCl	$T_d = 56 - 3.5 [\text{M GuCl}]$
CsTFA	$T_d = 72 - 2.5 [\text{M CsTFA}]$

*Values are valid for 1 to 3.5 M solutions with the 19-mer UP041/UP541 ODN duplex. Values presented are in °C.

We have determined that the T_d values for ODN/ODN duplexes in the presence of various chaotropes at concentrations from 1 to 3.5 M (Table 4) is a linear function⁵¹ given by the equation:

$$T_{d[\text{salt}]} = T_{d(0)} - k[\text{C}_x^-].$$

$T_{d(0)}$ is the extrapolated T_d at zero salt concentration, k is the chaotrope-specific constant and $[\text{C}_x^-]$ is the concentration of the chaotrope. In general, we have found that the slope (k) remains constant for any given sequence and length of duplex, but that the intercept value ($T_{d(0)}$) is sequence and length dependent (data not shown).

The use of chaotropic solutions must be tempered with the recognition that some anionic species may be chemically reactive. The nucleophilicity of the SCN^- anion is about 50% that of the sulfhydryl group,⁵² and therefore may pose unanticipated problems^{53,54} in diagnostic assays in some instances (i.e., when utilizing solid supports, especially with complex biological samples).

Mismatching in Low Stringency Chaotropic Hybridization Solutions

An important conclusion of the analysis of the effect of chaotrope type on T_d is that small changes in the concentration of the chaotrope can effect large changes in T_d and, therefore, stringency. The large k values for TCA^- , SCN^- and GuSCN-based hybridization solutions (Table 4) are evidence of this. The requirement of a high degree of fidelity is paramount if the exquisite specificity of ODN probes is to be utilized. We have observed that even short (24-mer) complementary ODN sequences will form misaligned duplex structures when sub-stringent hybridization conditions are used. As a model system complementary ODN sequences were hybridized to the ODN-bead about 10-fold past the $\text{C}_{0t_{1/2}}$ in 1, 2 or 3 M GuSCN (3 M GuSCN is nearly stringent at 19°C for the UP041-ODN duplex). The resulting duplexes were analyzed for the extent of mismatching (Figure 2) by determining the T_d in 0.18 M NaCl. Three parameters indicate the degree of mismatching: the extent of hybridization as a function of stringency, the width of the melting profile and the T_d value. As indicated in Figure 2, fully 30% of the hybridized material is mismatched under non-stringent conditions (1 M GuSCN, 19°C, 43°C below T_d). Interestingly, there is a 30% increase in the mass hybridized in sub-stringent solutions (panel A, Figure 2). Under highly non-stringent conditions, it therefore appears that the rate of dissociation for improperly aligned sequences (of extremely low complexity, 19 nt) can approach the rate of dissociation for perfectly matched duplexes.

It is widely assumed that low stringency ODN probe hybridization followed by a high stringency wash can be used to establish specificity.¹⁰ However, the work presented here indicates that there are drawbacks to this approach when the nucleic acid targets possess large sequence complexity (i.e., genomic DNA). First, in low-stringency conditions, the maximal yield of perfectly base-paired duplexes *can not* be achieved, although the apparent yield may seem to be increased (Figure 2, panel A). Second, the effective concentration of ODN probe may be significantly reduced due to non-specific hybridization. Therefore, when ODN probes are employed in rapid assays, it is important to perform hybridizations under fully stringent conditions as previously suggested for long DNA or RNA probes.⁵⁵

Optimal Yield of Hybridization as a Function of T_d with Covalently Immobilized ODN Probes on the ODN-bead

Rapid sandwich assay formats (< 30 minutes) restrict the ability to achieve C_0t values of target nucleic acid capture that exceed the $C_0t_{1/2}$ more than one-fold. A hybridization step in which target nucleic acid (assume for example rRNA, with $C_0t_{1/2}$ of capture of 5 minutes) is captured onto the ODN-bead is limited to 5–10 minutes in a 30 minute colorimetric assay.¹³ Therefore, the maximum rate of hybridization must be achieved to optimize the signal. Since the general rules developed for finding the optimal rate of DNA reassociation or hybridization do not apply to ODN probes, we have measured the yield of duplex formation as a function of T_d for tethered ODN probes. Although the optimal rate of reassociation has been shown to occur 20–25°C below the T_m for long DNA,⁵⁶ (in solution, the T_m of perfectly base-paired RNA/DNA hybrids is 10–15°C lower than the T_m of corresponding DNA/DNA duplexes in solutions not containing formamide or chaotropic salts^{57–62}), we show here that short ODN probes covalently immobilized on a solid support (ODN-bead) reach their optimal yield of hybridization at a temperature about 5°C below the T_d .

As shown in Table 5, the optimal temperature of hybridization which results in the maximal yield of hybridized product (T_{opt}^{ODN}), (either 16S rRNA or complementary ODN), occurs at about 5°C below the T_d of the respective duplex or hybrid structure. The T_{opt}^{ODN} was determined by measuring the extent of the reaction at the $C_0t_{1/2}$ of the reaction. Since the extent of the reaction was measured at $C_0t_{1/2}$, the extent of hybridization reflects the rate. For instance, the T_d of a 19 nt tethered UP041 ODN/ODN duplex in 0.18 M NaCl with a G + C content of 65% is 68°C, and the optimal temperature of hybridization is 63°C (Table 5). This T_{opt}^{ODN} is approximately the same temperature at which optimal DNA/DNA reassociation or RNA/DNA hybridization occurs in 0.12 to 0.18 M sodium phosphate in the case of long DNA or RNA probes.⁶³

This observation suggests that the temperature dependence of the forward rate of hybridization, *under the same salt or solvent conditions*, is in general, similar for both oligonucleotides and long probes. This is not surprising since duplex formation of nucleic acids is dependent on the nucleation frequency.⁵¹ Referencing the optimal temperature of hybridization to T_m or T_d with either long nucleic acid or ODN probes factors out length dependence, base composition and base sequence. (It has been shown that for ODNs, duplex stability is dictated by base sequence rather than composition).² With ODN probes, the apparent forward rate of hybridization decreases when the reverse reaction (dissociation or denaturation) begins to predominate. The

Figure 2. Mismatching of ODN duplexes in low stringency chaotropic hybridization solutions. As a model system to test stringency in a chaotropic hybridization solution, the ODN sequence UP541 was covalently immobilized to the ODN-bead¹³ and complementary ³²P-ODN (UP041) was hybridized in 1, 2, or 3 M GuSCN. The solid supports were subsequently washed in the respective hybridization solution and thermally melted in 0.18 M NaCl solution. The data were plotted in two ways to permit visualization of the extent of mismatching in low stringency solutions. The cumulative cpm at each temperature is plotted against temperature (panel A), and the % cumulative single strand material dissociated at each temperature was plotted versus temperature (panels B and C). About 30% of the total hybridized material is mismatched in 1 M GuSCN (cpm eluted from 34°C to 56°C, panel B). This is calculated by the ratio of ODN mass that hybridized in 1 M GuSCN to the ODN mass that hybridized in 3 M GuSCN (panel A). The melting profile of mismatched sequences spans a 40°C range, while the profile for properly matched sequences spans a 20°C range (panel C).

reverse reaction generally becomes predominant at 2–5°C below the T_d or T_m of the ODN duplex or hybrid depending on the ODN sequence. With 'long' DNA/DNA or RNA/DNA duplexes or hybrids, the reverse reaction (thermal instability leading to dissociation) occurs also near the T_m or T_d (2–5°C below the T_d or T_m) of the respective duplex structure. However, thermal instability with long probes occurs 15–20°C above the optimal temperature of DNA/DNA renaturation, or 10–15°C above the optimal temperature of hybridization for RNA/DNA. Therefore, it is inappropriate to apply the rule developed for sheared native DNA ($T_m - 25^\circ\text{C}$)^{38,64} to determine the maximum rate of renaturation or hybridization for ODN probes. It should be emphasized however that the $T_m - 5^\circ\text{C}$ rule for ODNs is only a guideline for searching for the optimal rate of hybridization with tethered ODN probes as each individual sequence will possess its own temperature optimum ($T_m - 2 - 8^\circ\text{C}$, data not shown). As demonstrated above (Figure 2), at temperatures 25°C

Table 5. A comparison of the optimal temperature of hybridization ($T_{\text{opt}}^{\text{ODN}}$) for ODN probes covalently immobilized on ODN-beads and the optimal rate of hybridization for long probes ($T_{\text{opt}}^{\text{LP}}$)

Duplex Type (Long Probes)	Solution	Length of duplex	T_m	$T_{\text{opt}}^{\text{LP}}$	$T_m - T_{\text{opt}}^{\text{LP}}$	Ref.
DNA/DNA	0.18M NaCl	400 nt	88–90	65	25	63
RNA/DNA	0.18M NaCl	400 nt	76	65	11	63
DNA/DNA	3 M GuSCN	100–1000 nt	56	37	19	7
RNA/DNA	3 M GuSCN	100–1000 nt	57	37	20	7
(Oligodeoxynucleotides)			T_d	$T_{\text{opt}}^{\text{ODN}}$	$T_d - T_{\text{opt}}^{\text{ODN}}$	
ODN/ODN	0.18M NaCl	19 nt	68	63	5	
ODN/ODN	3 M GuSCN	19 nt	38	33	5	
RNA/ODN	3 M GuSCN	19 nt	38	33	5	

below the T_d , adequate stringency can not be achieved with tethered ODN probes. An interesting feature of ODN probes is that it is possible to design and use an ODN probe under conditions in which the hybridization is occurring at a temperature which exceeds the T_m or T_d of the respective probe and its complementary nucleic acid target.

Comparison of T_m Values Obtained in Solution and T_d Values Measured Using Nylon Membranes or the ODN-bead Solid Support

The typical difference (7–8°C) between T_m and T_d was observed for duplexes on either nylon membranes or the ODN-bead as compared to T_m values obtained in solution. However, there exists a substantial difference between the ΔT_d values for nylon membranes and for the ODN-bead which affects conditions (temperature, washing times, etc.) required to achieve stringency.

The effect of chaotropic hybridization solutions on the T_d of ODN duplexes (shown above) was determined by dissociating duplexes from ODN-beads onto which one complementary sequence had been covalently attached (reference 13, and Appendix 1). It has been shown that a significant difference exists between the T_m of duplexes measured in solution and T_d values that are obtained by melting duplexes from nylon membranes or nitrocellulose.^{2,3,4} T_m values were empirically determined to be 7.6°C higher than T_d values (using nylon membranes).⁴ When the thermal transitions of duplex structures formed on solid supports (such as nylon membranes, nitrocellulose, or the ODN-bead) are measured, as described in Appendix 1, re-hybridization is not possible. Therefore, the thermal transition curve essentially represents an extrapolation to zero concentration of the dissociated complementary sequence during the dissociation process. Thermal transitions determined in solution (T_m s) are generally measured at a fixed (approximately 1–10 micromolar) equal molar concentration of complementary sequences. Thus, re-hybridization after melting is possible, accounting for the observed T_m dependence on concentration. The thermal transitions of identical duplexes in solution and on two types of solid supports are shown in Table 6.

The difference between the T_m values determined in solution and the T_d s measured with either ODN-bead (covalently immobilized capture sequence) or nylon filters (non-covalent immobilization of complementary sequence) was 6 to 7°C, in agreement with previously established values.⁴ Interestingly, however, the ΔT_d was 6 to 8°C larger when the T_d^{mem} values were determined using nylon membrane supports. This has been a consistent observation when ΔT_d values have been compared

Table 6. The effect of solid supports on T_d and ΔT_d .

Condition	Solution	T_m	ΔT_m
Solution ^o	3 M NaSCN	56 C	12 C
Solution ^o	3 M GuSCN	44.5 C	12 C
		T_d or T_d^{mem}	ΔT_d
Bead Support ^o	3 M NaSCN	48 C	12 C
Bead Support ^{o,*}	3 M GuSCN	38 C	12 C
Nylon Filter [*]	3 M GuSCN	37 C	18–20 C

T_m s, T_d^{mem} , and T_d s were determined using an ODN/ODN duplex^o (UP041/UP541) or a DNA/ODN duplex^{*} (*Pg* genomic DNA/UP041 ODN).

for nylon membranes and the ODN-bead (data not shown). This observation has two important implications. First, stringency is affected by the magnitude of the ΔT_d . Although the empirically determined T_d s for ODN duplexes or hybrids obtained using ODN-bead support and nylon membranes are nearly the same (Table 6), a higher final temperature (or longer or more numerous washes) is required to completely dissociate duplexes from the nylon membrane, since the ΔT_d^{mem} for nylon membranes is larger than the ΔT_d . Second, since the ΔT_d for the ODN-bead is the same value as the ΔT_m obtained in solution, we infer that the ODN-bead surface properties do not affect the dissociation of complementary sequences in contrast to the positively charged and porous surface of the nylon membrane. This is postulated to be due to the covalent immobilization of the ODN through long spacer linkages, and the non-porous surface character of the ODN-bead which possesses a net negative to zwitterionic charge.

Reduction of Background in a Sandwich Assay Format Using TCA-Based Hybridization Solutions

In a sandwich assay format, high concentrations of capture and signal ODN are utilized to create pseudo-first order conditions. For example, a capture ODN (covalently immobilized on an ODN-bead¹³) is employed in large excess over target nucleic acid and signal ODNs are generally employed at concentrations of about 1 $\mu\text{g/ml}$. However, at these concentrations, there is a 10^5 - to 10^6 -fold excess of signal ODN molecules over the lowest detectable level of biotinylated signal ODN (data not shown). Therefore, even infrequent interaction (mismatching, greater than 1 part in 10^6) of signal and capture ODNs will give rise to a significant background. Unexpectedly, we have noted that approximately 30% of the capture/signal ODN pairs that we have

Table 7. Non-specific interactions of capture and signal ODN in GuSCN and LiTCA hybridization solutions

Duplex Pair		Solution	Signal Strength*
Pg001/Pg501	(+ control)	GuSCN	++++
Pg001/UP007	(- control)	GuSCN	-
Pg001/UP003		GuSCN	++++
Pg001/Pg501	(+ control)	LiTCA	++++
Pg001/UP007	(- control)	LiTCA	-
Pg001/UP003		LiTCA	-

*Signal strength is based on quantity of Horse-Radish Peroxidase-generated product in sandwich assay format.¹³ Hybridization was performed at 19°C.

tested in the sandwich assay format show high levels of non-specific background (data not shown). A specific example is described below.

The capture/signal ODN pair Pg001/UP003 (Table 1) shows significant levels of background due to non-specific base-pairing in 3 M GuSCN, although the maximum number of base pairs that can form are 12/19 nucleotide bases (based on computer analysis).⁴ In the experiment described in Table 7, the Pg-specific sequence Pg001 was covalently immobilized on the ODN-beads¹³ which were then incubated with either the biotinylated complementary sequence (positive control), a biotinylated sequence which was previously established not to cause background (negative control), and biotinylated UP003. Two stringent hybridization solutions (stringency adjusted for the capture of an RNA target, Table 3) containing either GuSCN or LiTCA were compared in a colorimetric assay. As described in Table 6, significant levels of non-specific background were observed with the Pg001/UP003 ODN combination in GuSCN-based hybridization solutions.

As shown above, in GuSCN hybridization solutions the T_d of ODN/ODN duplexes is similar to that of corresponding RNA/ODN hybrids while in LiTCA hybridization solutions the T_d of ODN/ODN duplexes is 9°C below that of corresponding RNA/ODN hybrids (Table 3). In the LiTCA hybridization solution, no background was observed for the Pg001/UP003 ODN combination (Table 7). This is due to the preferential ability to disrupt infrequent mismatches between capture ODN and signal ODN sequences in LiTCA hybridization solutions. Thus, it is possible to capture an RNA target significantly above the approximate T_d of mismatched capture/signal ODN probe combinations. If the appropriate chaotrope is selected for particular capture probe/target/signal probe combinations (ODN/DNA/ODN, ODN/RNA/ODN, etc.), significant advantage can be obtained in terms of background reduction.

CONCLUSIONS

There are a number of parameters that affect the T_d of an ODN probe employed in an hybridization assay. These parameters include pH, G + C content, anion and cation type and concentration, length of duplex, duplex type, the degree of fidelity of base pairing, and the type of solid support employed. We have addressed here the effect of anionic and cationic species type on T_d and stringency. A stringent, ambient temperature hybridization may be performed if both the properties of the chaotropic solution, and the nature of the solid support are understood.

Figure 3. The effect of volume of the 'melting' solution on T_d . ODN sequence Pg001-derivatized ODN-beads were hybridized with complementary ³²P-ODN (Pg501) under stringent hybridization conditions (3 M GuSCN, 25°C). The ODN-beads were washed with the hybridization solution and then placed in either 0.1 ml, 0.2 ml, 0.4 ml, 0.8 ml, or 1.6 ml of 3 M GuSCN. At 5 minute intervals, the temperature was raised 5°C. The ramp time for the 5°C increment was about 1.2 minutes. The solution was decanted into a scintillation vial, and fresh hybridization solution (5°C below the next increment) was added to the beads. The T_d melt was conducted over a temperature range of 20°C to 65°C. The % cumulative single strand material dissociated at each temperature was plotted versus temperature. The point at which 50% of the material is eluted from the bead is taken as the T_d value.

Figure 4. The effect of the duration of the temperature interval on T_d . The conditions employed were identical to those described in Figure 3. The volume of the 'melting' solution was 0.4 ml. The times indicated in the Figure 4 include the ramp time to achieve a 5°C increase in temperature.

We have shown here that the T_d of an ODN/ODN duplex can be altered by as much as 90°C by changes in the both the concentrations and types (taxigenic to chaotropic) of hybridization salts. In high concentrations of TCA-based solutions, the T_d of an ODN/ODN duplex is below 0°C and in high concentrations of NaCl, the T_d is well above 80°C. Therefore, if the length, G + C content, and nucleic acid type are known, the chaotrope type and appropriate concentration can be chosen to permit ambient temperature hybridization based on the information presented here.

It has been argued that chaotropic salts decrease the activity coefficients of purine and pyrimidine bases (thus increasing solubility of the bases) and thus potentiate the thermal instability of nucleic acid duplexes.^{65,66} It may be possible therefore to develop a theoretical basis for predicting T_m or T_d values of

Figure 5. The effect of covalently immobilized ODN mass/ bead on T_d . Pg001-derivatized nylon beads were hybridized with either ^{32}P -Pg501 ODN complementary probe (Panel A), or, complementary rRNA in the presence of ^{32}P -UP041 ODN signal probe (Panel B) under stringent hybridization conditions in 3 M GuSCN. The ODN-beads were washed with the hybridization solution and then placed in 0.4 ml of 3 M GuSCN. At 5 minute intervals, the temperature was raised 5°C. The solution decanted into a scintillation vial, and fresh hybridization solution (5°C below the next increment) was added to the beads. The T_d values for each type of bead were determined concurrently for each target type.

ODN duplexes in chaotropic salt solutions if the activity coefficients or solubilities of the bases can be measured in chaotropic solutions, and then coupled with the recent advances^{2,3,4} in predicting T_m or T_d based on the sequence of ODNs.

Two possible complications are associated with the use of ODN probes for multi-target analysis in a sandwich assay format. First, the T_d of the capture probe and the T_m of signal probe must be very similar with the respective nucleic acid target. The T_d of the complete sandwich of capture ODN/target/signal ODN should be around 5°C above the hybridization temperature to attain high fidelity and the optimum rate of hybridization. Second, the complete sandwich assay T_d must be about the same (within 2–3°C) for each target nucleic acid that is to be simultaneously captured and detected. With the careful selection of probe length, chaotrope type, and concentration, an operational multi-target sandwich assay can be designed using ODN probes covalently immobilized on a solid support.

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Appendix 1. Parameters Affecting T_d Measurements with ODN-beads

T_d Determinations are Independent of the Volume of the Melting Solution

To demonstrate that the T_d values determined from the ODN-beads are independent of the volume of the 'melting' solution, the thermal dissociations steps were performed in volumes ranging from 0.1 ml to 1.6 ml, as shown in Figure 3. There is no discernable difference in the measured T_d values when the volume is increased from 0.1 ml to 1.6 ml. The 3/32nd inch diameter ODN-bead occupies about 0.0071 cm³. The results indicate that the dissociated complementary sequence is sufficiently dilute to preclude re-hybridization and the reported T_d values are independent of duplex or dissociated complementary sequence concentration using conditions described in the Methods section.

Time Dependency of the Dissociation Process

The length of time that the ODN-bead is held at each temperature during the dissociation process is shown to have a minimal impact on the measured T_d values. The time that duplexes were allowed to dissociate at each incremental temperature (5°C) was varied

from 2.5 minutes to 10 minutes (Figure 4), which includes the ramping time of 1.2 minutes. At the longest interval time of 10 minutes, the apparent T_d was decreased about 2°C, but at 2.5 or 5 minute intervals, the T_d s were nearly identical. The results indicate that the T_d value dependency on the time interval is minimal. For comparative studies, it would appear essential that all temperature intervals be constant. All measurements described in this report were performed using a time interval of 5 minutes.

The Effect of Capture Probe Concentration on T_d

The quantity of capture probe that can be covalently immobilized on the ODN-bead surface can be controlled and varied from 0.01 to 1.5 µg.¹³ The effect of the concentration of immobilized probe on the T_d has been measured for both the hybridization of ODN and rRNA on the ODN-bead at 5 different concentrations of capture ODN. The T_d of hybridized complementary ODN varied from 34°C with 0.06 µg/bead to 36.5°C with 1.1 µg/bead (Panel A, Figure 5). When the captured target nucleic acid was rRNA, the T_d difference was about 2°C between 0.06 µg/bead and 1.1 µg/bead (Panel B, Figure 5). The ODN-beads used in this study had 0.12 to 0.2 µg/bead of immobilized ODN. Although the differences in values of T_d as a function of ODN loading are small, the same loading level should be used when direct T_d comparisons are made.