



Strategies for Attaching Oligonucleotides to Solid Supports

Contents

1. Introduction.....	1
2. Two-dimensional Surfaces (Microarray slides).....	2
2.1 Surface Treatment.....	3
3. Three-dimensional Surfaces (Micro-spheres).....	4
3.1 Handling Fluorescent Micro-spheres	5
3.2 Attachment:.....	5
3.3 Applications.....	6
3.3.1 Liquid Arrays.....	6
3.3.2 Detection of Single Nucleotide Polymorphisms.....	7
4. Modifications.....	7
4.1 I-Linker.....	7
4.2 Amino-modified Oligonucleotides	7
4.2.1 5' Amino Modifiers.....	8
4.2.2 3' Amino Modifiers.....	8
4.2.3 Internal Amino Modification.....	8
4.2.4 Labeling Amino Modified Oligonucleotides.....	9
4.2.5 Attaching Amino-Modified Oligonucleotides.....	9
4.3 Thiol-Modified Oligonucleotides.....	12
4.3.1 5' Thiol Modifier	13
4.3.2 3' Thiol Modifier	13
4.3.3 Storing and Reducing Thiol Modified Oligonucleotides.....	13
4.3.4 Attaching Thiol Modified Oligonucleotides.....	15
4.4 Acrydite™-Modified Oligonucleotides.....	16
4.4.1 Advantages of Acrydite chemistry	16
4.4.2 Acrydite Oligos from IDT	17
4.4.3 Attaching Acrydite-Modified Oligonucleotides.....	18
5. Conclusion	19
6. References.....	20

1. Introduction

Many important molecular applications, such as DNA oligonucleotide arrays, utilize synthetic oligonucleotides attached to solid supports. The most accessible approach for producing an oligonucleotide microarray is to synthesize individual oligonucleotides and subsequently immobilize them to a solid surface. For this immobilization to take place, the oligonucleotides

must be modified with a functional group in order to have attachment to a reactive group on a solid surface.

Oligonucleotides can be attached to flat two-dimensional surfaces, such as glass slides, as well as to three-dimensional surfaces such as micro-beads and micro-spheres. Construction of arrays involves a number of parameters each of which must be optimized for efficient and effective experimental design.

Here, we present information on attachment to two- and three-dimensional surfaces as well as discuss the more commonly used chemistries available for solid support attachment.

Modifications available at IDT include for oligonucleotide attachment include:

- I-Linker™
- Amine-modified oligos covalently linked to an activated carboxylate group or succinimidyl ester
- Thiol-modified oligos covalently linked via an alkylating reagent such as an iodoacetamide or maleimide
- Acrydite™-modified oligos covalently linked through a thioether
- Digoxigenin NHS Ester
- Cholesterol-TEG
- Biotin-modified oligos captured by immobilized Streptavidin

2. Two-dimensional Surfaces (Microarray slides)

Substrates for arrays are usually silicon chips or glass microscope slides. Glass is a readily available and inexpensive support medium that has a relatively homogeneous chemical surface whose properties have been well studied and is amenable to chemical modification using very versatile and well developed silanization chemistry [1]. Most attachment protocols involve chemically modifying the glass surface to facilitate attachment of the oligo. Silianized oligonucleotides can also be covalently linked to an unmodified glass surface [1].

Amino, thiol, and Acrydite™ modifications have been routinely used to construct oligonucleotide arrays. Construction of arrays involves a number of parameters each of which must be optimized for efficient and effective experimental design. One important parameter is the method of choice for attachment of the synthetic oligonucleotides. Some of the issues to be considered in choosing an appropriate support and the associated attachment chemistry include: the level of scattering and fluorescence background inherent in the support material and added chemical groups; the chemical stability and complexity of the construct; the amenability to chemical modification or derivatization; surface area; loading capacity and the degree of non-specific binding of the final product [1]. Different modifications allow immobilization onto different surfaces:

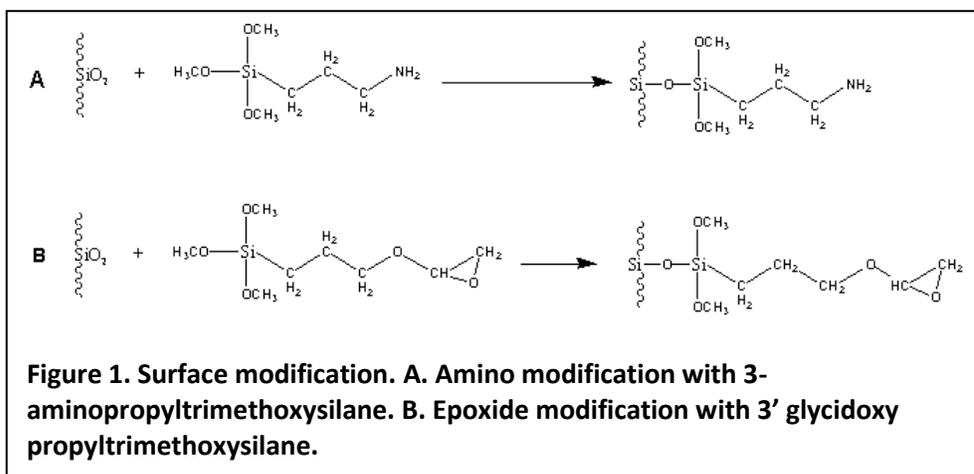
Modification	Surface treatment
--------------	-------------------

NH ₂ -modified oligos	Epoxy silane or Isothiocyanate coated glass slide
Succinylated oligos	Aminophenyl or Aminopropyl-derivatized glass slide
Disulfide modified oligos	Mercaptosilanized glass support
Hydrazide (I-Linker™)	Aldehyde or Epoxide

2.1 Surface Treatment

The two-dimensional surface is typically prepared by treating the glass or silicon surface with an amino silane which results in a uniform layer of primary amines or epoxides (Figure 1) [1, 2]. These modifications meet several criteria [3]; the linkages are chemically stable, sufficiently long to eliminate undesired steric interference from the support, and hydrophilic enough to be freely soluble in aqueous solution and not produce non-specific binding to the support [1]. Once these modifications have activated the surface, the efficiency of attaching the oligonucleotides depends largely on the chemistry used and how the oligonucleotide targets are modified [4].

Oligonucleotides modified with an NH₂ group can be immobilized onto epoxy silane-derivatized [5] or isothiocyanate coated glass slides [1]. Succinylated oligonucleotides can be coupled to aminophenyl- or aminopropyl-derivitized glass slides by peptide bonds [6], and disulfide-modified oligonucleotides can be immobilized onto a mercaptosilanized glass support by a thiol/disulfide exchange reactions [7] or through chemical cross linkers.



The surface density of the oligonucleotide probe is expected to be an important parameter of any array. A low surface coverage will yield a correspondingly low hybridization signal and decrease the hybridization rate.

Conversely, high surface densities may result in steric interference between the covalently immobilized oligonucleotides, impeding access to the target DNA strand [1]. Guo et al. found an optimal surface coverage at which a maximum amount of a complementary PCR product is hybridized [1]. In a series of hybridization studies using PCR products as probes these authors determined the optimal surface coverage at which a maximum amount of the complementary PCR product is hybridized. For a 157 base probe, the optimum occurred at about 5.0 mM

oligonucleotide concentration, which corresponded to a surface density of 500 Å²/molecule [1]. The optimum for a longer 347 nucleotide fragment was found to be approximately 30% lower, probably reflecting the greater steric interference of the longer fragment.

The planar surface structure of glass slides or silicon chips can limit the loading capacity of oligonucleotides. This limitation has been addressed by applying acrylamide gels to glass slides to construct a three dimensional surface, greatly increasing the surface area per spot [8, 9]. The increased surface area permits relatively large amounts of DNA to be linked to the surface resulting in strong signal intensities and a good dynamic range. This effect is partly offset by a higher background produced mainly by the same structural characteristic (the large surface area per spot) responsible for the high loading capacity [10].

3. Three-dimensional Surfaces (Micro-spheres)

Molecular biologists and chemists are discovering that micro-spheres are ideal substrates for an ever-increasing number of applications that use synthetic oligo-nucleotides. In these micro-sphere-based assays, each oligonucleotide is attached to a micro-sphere. The micro-spheres can be individually assayed, usually with a flow cytometer, or isolated based on the physical characteristics of the bead. They are amenable to multiple assay formats, multiplexing, rapid assay development and have distinct cost advantages over two-dimensional glass or silicon chip arrays for characterizing populations of nucleic acids. Several different types of micro-spheres are available:

Polystyrene micro-spheres can be fluorescently labeled through internal dye entrapment or surface attachment [11]. Surface labeling is preferred if the environmental responsiveness of the dye is important or if the particles are to be used in a nonaqueous solvent. In most applications, however, internally labeled micro-spheres are favored. Internal labeling leaves surface groups available for coupling reactions. Additionally, internally labeled fluorescent micro-spheres are generally brighter, less susceptible to photo bleaching, and are available in a larger range of colors than surface-labeled micro-spheres. To entrap the fluorescent dye, the polymeric micro-spheres are swelled in an organic solvent or dye solution. The water-insoluble dye diffuses into the polymer matrix and is trapped when the solvent is removed from the micro-spheres. With these techniques, scientists at Luminex have been able to create a set of 100 unique fluorescent polystyrene micro-spheres simply by using precise ratios of two spectrally distinct fluorophores.

Magnetic micro-spheres have been used for many years in radioimmunoassays, ELISAs, and cell separation assays. Magnetic micro-spheres are synthesized by dispersing ferrite crystals in a suspension of styrene/divinylbenzene monomers and polymerizing this cocktail into micro-spheres. A popular use for this type of micro-sphere has been to capture mRNA from a cell lysate using beads coupled to oligo dT. These micro-spheres are often used to shorten the processing time in automated high-throughput systems [12]. The magnetic beads must be encapsulated for most molecular biology applications to ensure that the iron doesn't interfere with polymerases

and other enzymes. Polystyrene magnetic beads are available with both carboxylic acid and amine surface chemistries.

Silica micro-spheres have a relatively homogeneous chemical surface that can easily be modified using versatile and well developed silanization chemistry [1]. Their use in molecular biology has been primarily in solid phase reversible immobilization (SPRI) protocols for nucleic acid purification. Nucleic acids can be isolated and purified from a cell lysate or other solution by capture onto silica beads in the presence of a chaotropic agent (KI, NI, or NaSCN). The negatively charged nucleic acid adsorbs to the bead through an ionic interaction and is later eluted in low ionic strength buffer. SPRI purification is easy to automate and is often the method of choice in high-throughput sequencing facilities [13]. Silica micro-spheres are denser than polystyrene or latex micro-spheres and can easily be separated from liquid phase by gravity sedimentation or gentle centrifugation.

3.1 Handling Fluorescent Micro-spheres

Store fluorescent micro-spheres as aqueous suspensions at 2–8°C and in the dark to minimize photobleaching. Use aseptic techniques where practicable. Internally dyed micro-spheres should not be exposed to organic solvents since this will cause swelling of the polymer matrix and leaching of the dye. If aggregation is a problem, a surfactant such as Tween 20 may be added to the suspension.

3.2 Attachment:

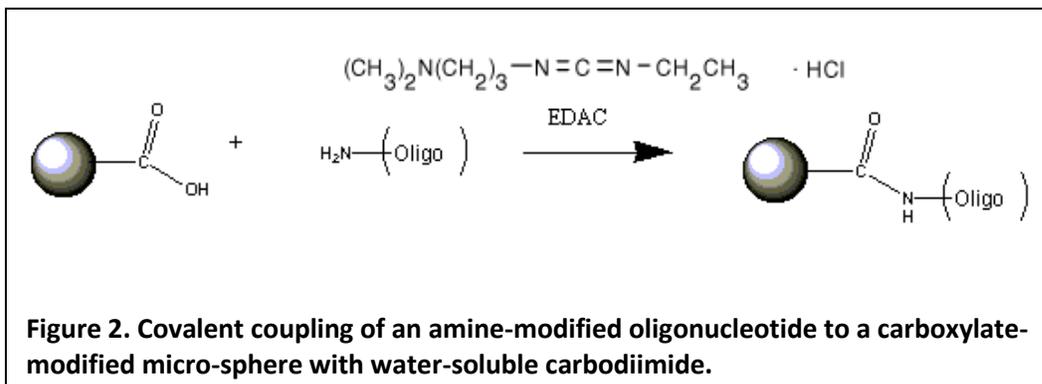
Nucleic acids can be covalently attached to micro-spheres with any of several methods. Carboxyl and amino groups are the most common reactive groups for attaching ligands to surfaces. These groups are very stable over time, and their chemistries have been widely explored. Listed below are several reactive groups that can be incorporated on the micro-sphere surface for covalent coupling.

Micro-sphere Surface Chemistries for Covalent Coupling

-COOH	Carboxylic acid
-RNH ₂	Primary aliphatic amine
-ArNH ₂	Aromatic amine
-ArCH ₂ Cl	Chloromethyl (vinyl benzyl chloride)
-CONH ₂	Amide
-CONHNH ₂	Hydrazide
-CHO	Aldehyde
-OH	Hydroxyl
-SH	Thiol
-COC-	Epoxy

Attaching an amino group to the 5' or 3' end of an oligonucleotide or a PCR primer is straightforward and inexpensive. These amine-modified oligos can then be reacted with

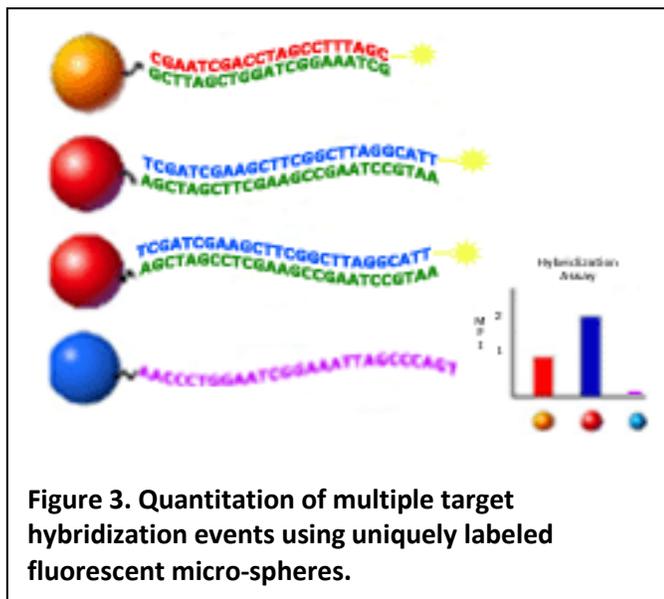
carboxylate-modified micro-spheres with carbodiimide chemistry in a one-step process at pH 6–8 (Figure 2). One typical water-soluble carbodiimide is 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride (EDAC). The chief advantage of EDAC is that it provides one-step coupling. Unfortunately, EDAC is indiscriminate; it can crosslink any two primary amines in the reaction. Using this reagent may yield a matrix of crosslinked oligos and micro-spheres. Another option is to bind biotin-labeled oligos to avidin-coated beads.



3.3 Applications

3.3.1 Liquid Arrays

The availability of large sets of fluorescent micro-spheres that can be used together allows for highly multiplexed assays and efficient sample processing. For example, using a set of 100 distinct fluorescent beads in a multiplex format could generate almost 10,000 unique data points from a single 96-well plate which is a scale normally associated with microarrays. One platform that exploits this potential is the Luminex LabMAP™ system. A capture probe or target-specific oligonucleotide is coupled to the surface of a fluorescent micro-sphere. A specific fluorescent dye combination is used to uniquely label each micro-sphere and serves to encode bead identity. These spectral "bar codes" are equivalent to the x,y positioning



information on a 2-D solid array. The target (another oligo, a PCR product, cDNA, or protein) is labeled to allow fluorescent detection and quantitation. Hybridization takes place in solution between the capture probe and denatured target. Hybridization in solution is much more efficient than solid-phase hybridization and this efficiency is one of the advantages of micro-sphere-based assays. Following hybridization, the micro-spheres are assayed by flow cytometric methods. Two signals are assayed: the unique fluorescent signal that identifies the probe and the fluorescent signal that indicates hybridization has occurred. Relative fluorescence intensity of the

hybridization signal is used to quantitate the target (Figure 3). Yang et al. recently described a bead-based liquid array for detecting gene expression in a high throughput assay [14]. Defoort and colleagues used bead-based flow cytometric methods and reverse transcription–PCR to simultaneously identify HIV, hepatitis B, and hepatitis C in a single plasma sample[15].

3.3.2 Detection of Single Nucleotide Polymorphisms

Single-nucleotide polymorphisms (SNPs) are the most common form of sequence variation between individuals [16]. Analysis of this variation offers an opportunity to understand the genetic basis of disease and is a driving force behind modern pharmacogenomics. Accurate, high throughput, and cost effective methods to analyze SNPs is crucial to fully utilize the medical value of the DNA sequence data that has been generated in the human genome project. Several methods for discerning SNPs have been described including oligonucleotide ligation assay (OLA) [16], single base chain extension assay [17, 18], and allele-specific oligonucleotide hybridization (ASO) [19]. Each of these assays can be adapted to multiplex-analysis using arrays of oligonucleotides attached to fluorescently encoded microspheres [20]. An example of one of these bead-based adaptations, Allele specific primer extension (ASPE) is illustrated in Figure 4.

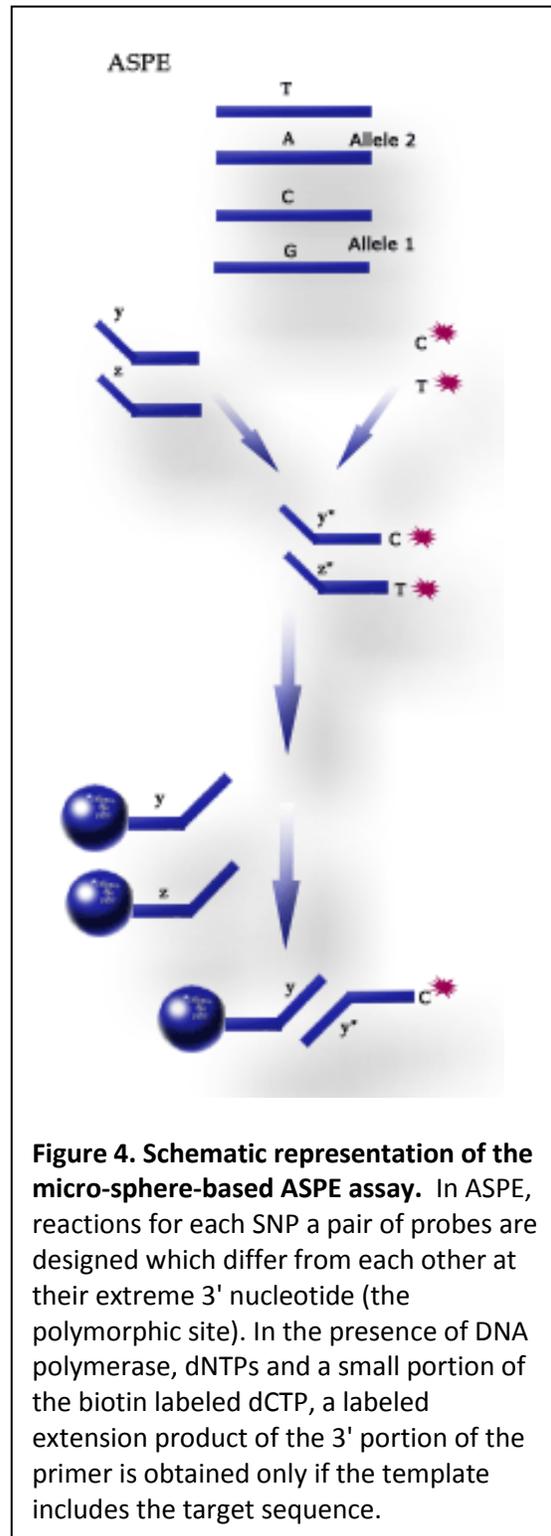


Figure 4. Schematic representation of the micro-sphere-based ASPE assay. In ASPE, reactions for each SNP a pair of probes are designed which differ from each other at their extreme 3' nucleotide (the polymorphic site). In the presence of DNA polymerase, dNTPs and a small portion of the biotin labeled dCTP, a labeled extension product of the 3' portion of the primer is obtained only if the template includes the target sequence.

4. Modifications

4.1 I-Linker

I-Linker™ is a proprietary covalent attachment chemistry for oligonucleotides that was developed at IDT. The modifier is attached to the 5'-end of the oligo. I-Linker™ can be substituted for amino modifications in many applications. In addition, I-Linker™ expands the range of reactive groups that can be used for conjugation, including aldehyde- and ketone-modified ligands or surfaces.

4.2 Amino-modified Oligonucleotides

A primary amine can be used to covalently attach a variety of products to an oligonucleotide, including fluorescent dyes [21, 22], biotin [23], alkaline phosphatase [24], EDTA [25], or to a solid surface. An amino modifier can be placed at the 5'-end, 3'-end or internally using an amino-dC or amino-dT modified base.

4.2.1 5' Amino Modifiers

Various amino-modifiers can be added to the 5'-terminus of a target oligonucleotide. Because conventional automated synthesis proceeds from 3' to 5', addition of the 5'-amino-mod is the last step in synthesis. As a result, any failed products which are truncated and capped will not receive the 5'-amino-modification and will not participate in subsequent chemical reactions involving the primary amine.

5' amino modifiers are β -cyanoethyl phosphoramidites which, when activated with 1H tetrazole, can couple to the 5' terminus of the oligonucleotide in the same time frame and with similar efficiency as nucleoside phosphoramidites. A number of 5' amino modifiers are available from IDT, these include simple amino groups with a six or twelve carbon spacers, a Uni-Link Amino modifier or amino modified thymidine or cytosine.

The shorter carbon chain linkers (Amino C6 and Uni-Link) may be used to attach compounds where proximity to the oligonucleotide poses no problem. The longer carbon chain linker (Amino C12) is recommended whenever steric or charge considerations require greater distance between the oligo and ligand or surface; examples include some affinity chromatography applications where the oligonucleotide must be adequately spaced from the surface, labeling with biotin [23], or certain fluorescent tags where interaction with the oligonucleotide, or the duplex it forms, may partially quench fluorescence [21, 22]. Sometimes even greater distance is needed than can be achieved by the amino-C12 modifier. In this case, one or more internal spacer modifiers (such as the S18 spacer) can be used to further separate the 5'-terminal amino-modifier from the oligo.

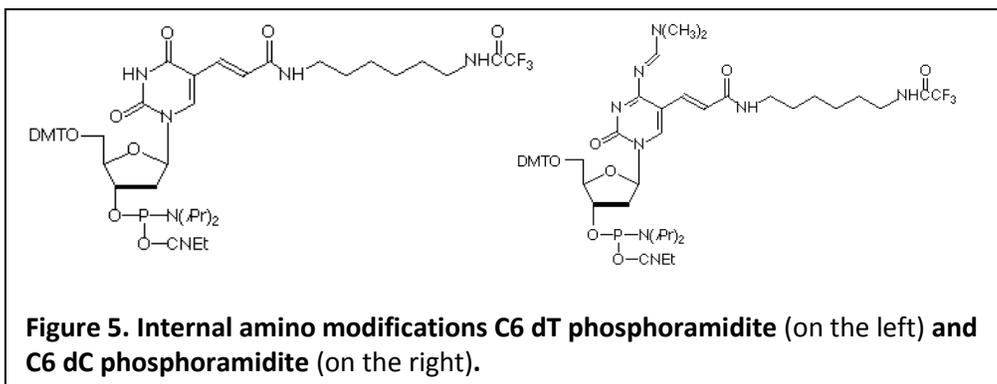
4.2.2 3' Amino Modifiers

3'-amino-modifiers contain branched linkers in which the amino group is protected with the fluorenylmethoxycarbonyl (Fmoc) group which was originally popularized for use in peptide chemistry [26, 27]. The Fmoc group is stable yet can be removed specifically from the support to allow solid-phase addition of an amino linked modification. However, during handling, some Fmoc groups can be lost; in this setting the free amino group is capped with acetic anhydride during synthesis and results in decreased yields.

3'-Amino-Modifier CPGs (the starting support for oligonucleotide synthesis) otherwise support oligo synthesis with the same efficiency as the standard nucleoside CPGs. After deprotection, the finished oligonucleotide has a free primary amine at the 3'-terminus. An oligo with a 3'-amino-mod can be labeled at the 5'-end with various phosphoramidite modifier groups (like fluorescent dyes, biotin, etc.) during synthesis. Alternatively, the oligo can be left with a free 5'-OH, which can later be labeled with ^{32}P using a polynucleotide kinase. The 3'-amino-modifier eliminates the native 3'-OH group from the oligo, which functionally blocks this oligo from participating as a primer in DNA synthesis, sequencing, or PCR.

4.2.3 Internal Amino Modification

Amino-modifier C6 dT and the recently introduced amino-modifier C6 dC are available for internal labeling (Figure 5). Addition of the amino-modifier itself does not adversely affect oligo hybridization characteristics; however subsequent addition of a bulky hydrophobic dye or other ligand can lower T_m or interfere with primer function. Therefore, it is preferable to attach large bulky groups to the 5'-end of the oligo, separated by spacers as needed. Amino-modifier dT and dC couple with similar efficiency as normal phosphoramidite monomers and their trifluoroacetyl-protecting group is removed during the standard ammonium hydroxide deprotection step.



4.2.4 Labeling Amino Modified Oligonucleotides

This general procedure can be used to conjugate amino-modified oligonucleotides with active succinimidyl ester or isothiocyanate derivatives of various ligands, such as fluorescent dyes. At pH 9.0 the conjugation reaction occurs virtually exclusively at the free primary amine and does not involve the exocyclic amino groups of the nucleosides.

1. For a 250 nmole scale synthesis, resuspend the amino-modified oligonucleotide (i.e. approximately 100 nmoles of primary reactive amine) in 0.7 mL of sterile distilled water.
2. Add 100 μ L of 10X conjugation buffer (1.0 M NaHCO₃)/Na₂CO₃, pH 9.0).
3. Freshly prepare a 10 mg/mL solution of active ester in DMF. Add 200 μ L of the solution to the reaction mixture.
4. Allow the mixture to stand at least 2 hours.
5. Remove the unreacted ligand by gel filtration (such as Sephadex G-25).
6. Depending on the reactivity of the NHS-ester used, coupling efficiency can range from 20-80%. Labeled-oligo can be purified from unreacted oligo by preparative RP-HPLC purification.

4.2.5 Attaching Amino-Modified Oligonucleotides

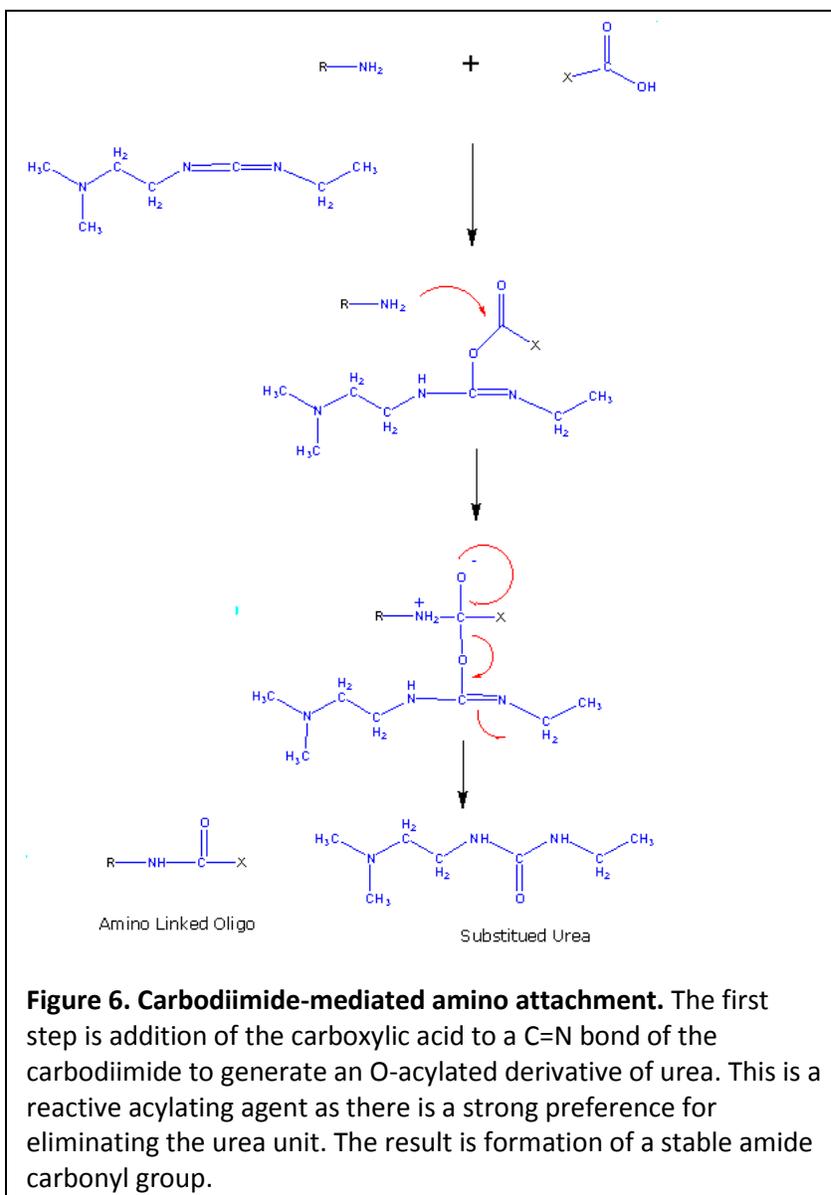
The attachment of an amino-modified oligonucleotide to a surface or another molecule requires an acylating reagent. Depending on which acylating reagent is used, carboxamides, sulfonamides, ureas or thioureas are formed upon reaction with the amine moiety. The kinetics of the reaction depends on the reactivity and concentration of both the acylating reagent and the amine. Buffers that contain free amines such as Tris and glycine must be avoided when using any amine-reactive reagent.

Attachment chemistries currently in use for amino modified oligonucleotides for linkage to molecule or surface:

Acylating Agent	Linkage	Features
Carbodiimide	Carbonyl amide	Most common method, stable attachment
Isothiocyanate	Thiourea	Stable covalent attachments
Sulfonyl chloride	sulfonamide	Sulfonyl chloride is unstable in water, but once conjugated to the oligo the sulfonamide bond is very stable
Succinimidyl esters (NHS-ester)	carboxamide	Carboxamide bond formed is very stable

The most significant factors affecting the reactivity of an amine are its class and its basicity. Oligonucleotides with an amino modification have a free aliphatic amine that is moderately basic and reactive with most acylating reagents. However, the concentration of the free base form of aliphatic amines that are below pH 8 is very low; thus, the kinetics of acylation reactions of amines by isothiocyanates, succinimidyl esters and other reagents are strongly pH dependent. A pH of 8.5-9.5 is usually optimal for oligonucleotide conjugation. Aromatic amines, which are present within each base, are very weak bases and, thus, are not protonated at pH 7 or above.

Most attachment chemistries currently in use for amino modified oligonucleotides utilize a carbodiimide mediated acylation. Acylation of a carboxyl group generates a stable amide carbonyl as illustrated in Figure 6. Isothiocyanates form thioureas upon reaction with amines that are relatively stable covalent attachments (Figure 7). A very common example of this reaction is the coupling of fluorescein isothiocyanate (FITC) with amino-labeled oligos

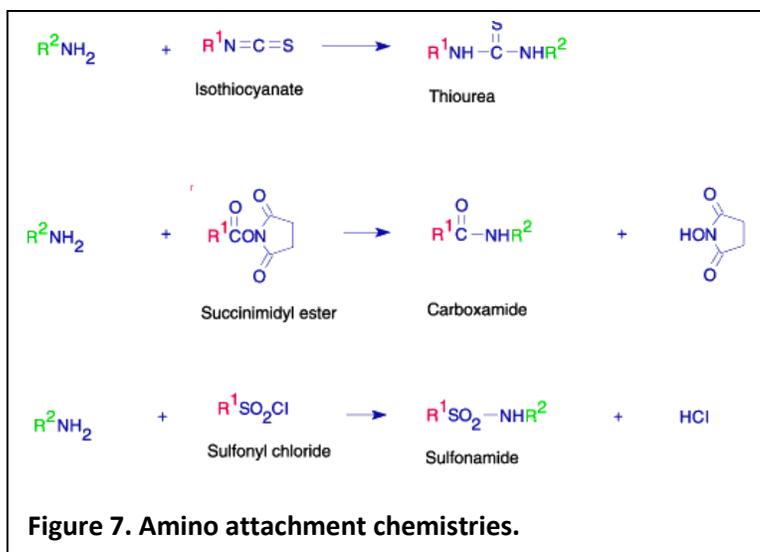


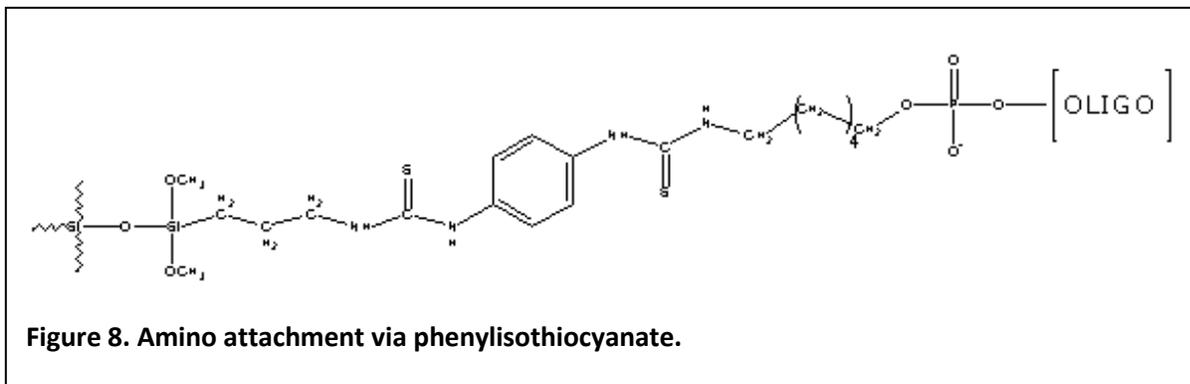
to form a fluorescent probe. Succinimidyl esters (NHS-esters) are excellent reagents for amine modification because the amide bonds they form (Figure 7) are very stable. These reagents can be stored long term if frozen or desiccated. NHS-esters are very reactive with primary amines and have low reactivity with secondary amines, alcohols, phenols (including tyrosine) and histidine. Succinimidyl esters will also react with thiols in organic solvents to form thioesters. Succinimidyl ester hydrolysis can

compete with conjugation, but this side reaction is usually slow below pH 9. Some succinimidyl ester derivatives, such as certain fluorescent dyes, have poor solubility in aqueous solution and must be resuspended in an organic solvent.

Sulfonyl chlorides are also reactive with primary amines. However, these reagents are quite unstable in water, especially at the higher pH required for reaction with aliphatic amines, and as such are not commonly used in oligonucleotide attachment protocols (Figure 7). Once conjugated, however, the sulfonamides that are formed are extremely stable. Sulfonyl chlorides can also react with phenols (including tyrosine), aliphatic alcohols (including polysaccharides), thiols and imidazoles.

As noted, a number of methods for attachment of 5'-amino-modified oligonucleotides to solid supports have been described. One method involves using an epoxide opening reaction to generate a covalent linkage between 5'-amino-modified oligonucleotides and epoxy silane-derivatized glass [5, 28]. Attachment of 5' amino-modified oligonucleotides on epoxy derivatized slides is relatively thermostable, but the sensitivity of the epoxy ring to moisture is a major drawback and leads to reproducibility problems [29]. More commonly, attachment of amino-modified oligos involves reacting the surface bound amino groups with excess p-phenylene 1,4 diisothiocyanate (PDC) to convert the support's bound primary amines to amino-reactive phenylisothiocyanate groups. Coupling of 5' amino-modified oligos reaction to the phenylisothiocyanate groups follows, resulting in the covalent attachment of the oligonucleotide (Figure 8) [1].





Modifications on this theme have included using homobifunctional crosslinking agents such as disuccinimidylcarbonate (DCS), disuccinimidylloxalte (DSO), and dimethylsuberimidate (DMS). These have all been used to covert glass bound amino groups into reactive isothiocyanates, N-hydroxysuccinimidy-esters (NHS-esters) or imidoesters, respectively. Activation with PDITC, DMS, DSC and DSO work well for the attachment of oligonucleotides, while immobilization of amino-linked PCR fragments cross-linking with PDITC or DMS is superior because they are less labile to the Tris buffer reagent in PCR amplification buffers [10].

The chemistry of 1-ethyl=3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, Figure 9) has been employed with other supports such as amino controlled-pore glass [30], latex beads [31, 32], dextran supports [33], and polystyrene [34]. Heterobifunctional cross-linkers such as EDC are designed to form stable covalent links in aqueous solution and their use in binding oligonucleotides onto glass surfaces by their 3' [35] or 5' [36] ends has been widespread.

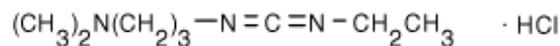


Figure 9. 1-ethyl=3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC).

4.3 Thiol-Modified Oligonucleotides

The thiol (SH) modifier enables covalent attachment of an oligo to a variety of ligands. A SH-modifier can be placed at either the 5'-end or 3'-end of an oligo. It can be used to form reversible disulfide bonds (ligand-S-S-oligo) or irreversible bonds with a variety of activated accepting groups. Options include active esters or isothiocyanate derivatives, as are commonly used for tagging free amino-modified oligonucleotides. Maleimide, bromide, iodide, or sulphonyl derivatives are suitable for tagging thiol-linked oligonucleotides with a variety of groups such as fluorescent dyes [21, 22], biotin [23] and alkaline phosphatase [24]. Conjugation of oligonucleotides to enzymes, such as alkaline phosphatase or horseradish peroxidase, is commonly employed in commercial probe systems. The thiol modification also enables attachment to solid surfaces (e.g. CPG-SH; Pierce) via a disulphide bond [37] or maleimide linkages.

Thiol-modifiers can be incorporated at the start of synthesis by placing the reactive SH-group at the 3'-end using a thiol-CPG and can also be incorporated as the last step of synthesis using a thiol-phosphoramidite which places the reactive SH-group at the 5'-end of the oligo.

4.3.1 5' Thiol Modifier

The incorporation of a thiol group at the 5' end of an oligo is achieved with S-trityl-6-mercaptohexyl derivatives (Figure 10) [21, 38]. IDT recommends HPLC purification of thiol-modified oligonucleotides.

4.3.2 3' Thiol Modifier

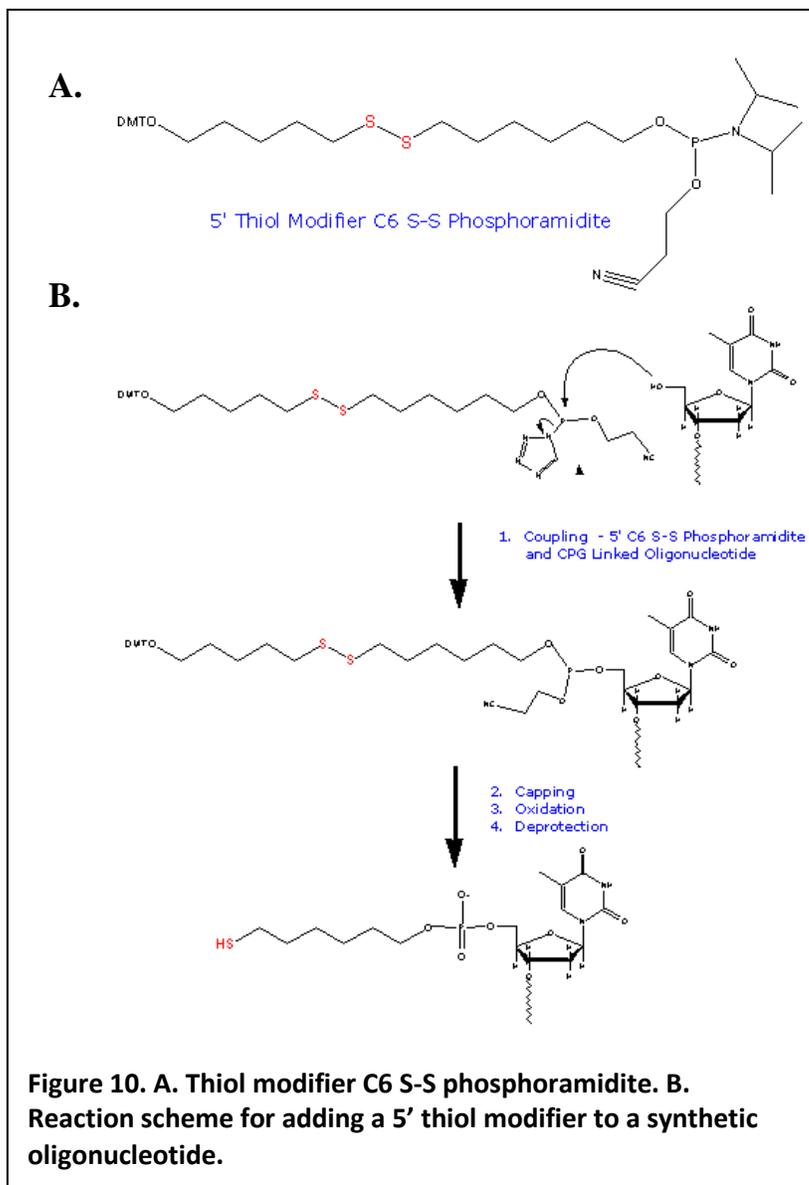
The 3'-thiol modifier C3 S-S CPG is used to introduce a thiol group to the 3'-terminus of a target oligonucleotide. A 3' thiol group can be particularly useful for post-synthesis modification in cases where a second 5' modification is already in place. Special precautions are taken during the oxidation steps of the synthesis to avoid oxidative cleavage of the disulfide linkage. Deprotection with a cocktail containing DTT is used to remove the base protecting groups and cleave the disulfide linkage to generate the reactive 3'-thiol.

Oligos from IDT are provided in a disulfide form (Figure 11). However, even when shipped in the disulfide form, spontaneous cleavage will occur to some variable fraction of the molecules. This is normal and will cause disulfide dimers to accumulate over time. Because of this, all thiol-oligos need to be reduced in preparation prior to conjugation immediately before use (see protocols below).

4.3.3 Storing and Reducing Thiol Modified Oligonucleotides

Thiol-modified oligonucleotides should be stored frozen. To ensure full reactivity, thiol-modified oligos should be reduced immediately before use. In general, the oligo is treated with a reducing agent (like DTT) and this agent is fully removed prior to coupling. Specific protocols are provided below.

4.3.3a Reduction Protocols:



Protocol 1. Treatment with solid-phase DTT

DTT is available immobilized on acrylamide resin (Reductacryl, Calbiochem Inc. Cat. No. 233157). The Reductacryl reagent can be used in a batch technique to reduce the disulfide bonds.

1. Resuspend oligo plus resin in TE (or similar buffer which is neutral or slightly alkaline such as pH 7.5).
2. Use a ratio of 1 mg oligo with 50 mg resin to ensure complete reduction.
3. Stir or agitate at room temperature for 15 minutes.
4. Remove Reductacryl™ by filtration (e.g., pass through a syringe filter)
5. Activated oligo can be directly injected into the coupling reaction or can be stored for brief periods of time before use.

Protocol 2. Treatment with DTT in liquid phase

The oligo can be treated with DTT or stored in DTT, which must be removed immediately before use.

1. Make a solution of oligonucleotide in TE plus DTT. We use 100 μ M oligo in 10 mM DTT in 1x TE.
2. Pass oligo through a large bed volume Sephadex column to remove DTT. Note that small bed volume spin columns can allow trace DTT to remain with the oligo, which can interfere with subsequent coupling reactions. As an alternative, use the extraction procedure outlined below.

Protocol 3. Bulk Reduction

Reconstitute the oligonucleotide (up to 1 mg of oligo can be used) in 100 μ L of 2% TEA (triethylamine), 50 mM DTT and allow to stand at room temperature for 10 min. Remove DTT using one of the 3 methods outlined below:

1. Extraction/PPT: extract 4x using 400 μ L of ethyl acetate (layers readily separate and the DTT will partition with the ethyl acetate and the DNA will partition in the aqueous phase).
2. Recover oligo by acetone precipitation or gel filtration.
 1. Add five volumes of acetone solution (2% LiClO₄ w/w in acetone) to one volume of the oligo solution in a 14 mL tube.
 2. Chill the resulting solution at -20°C for 15 minutes.

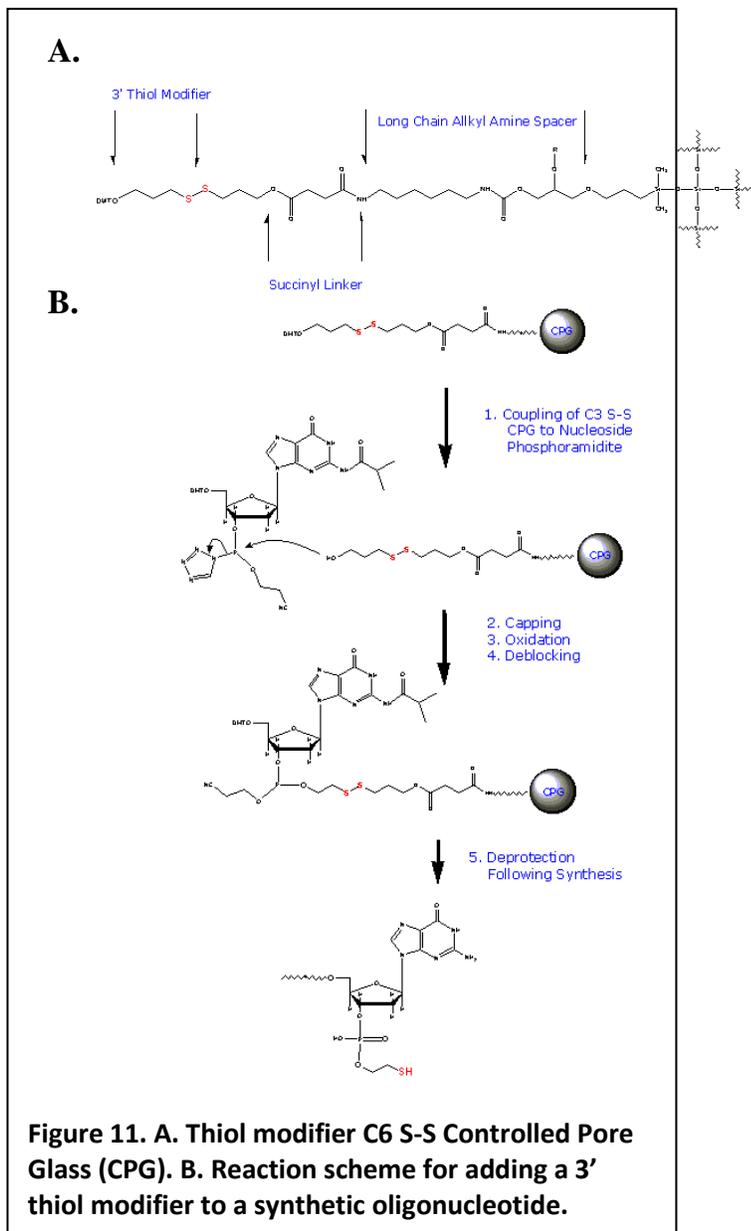


Figure 11. A. Thiol modifier C6 S-S Controlled Pore Glass (CPG). B. Reaction scheme for adding a 3' thiol modifier to a synthetic oligonucleotide.

3. Centrifuge the sample at 2500 - 5000 RPM for 10 - 5 minutes respectively.
 4. Remove the supernatant.
 5. Dry the sample under vacuum to remove trace acetone.
 6. To remove LiClO_4 and other salts, the sample can be washed with 2-3 mL of n-butanol centrifuged again followed by removal of the butanol supernatant.
3. Size exclusion or gel filtration chromatography
 1. Load the sample of oligonucleotide on a Sephadex G25F column that has been thoroughly washed with distilled water.
 2. Elute the column with water by gravity flow and collect fractions.
 3. Measure the UV absorbance at 260 nm. The first eluting peak at the void volume is the oligonucleotide.
 4. Concentrate fractions using a SpeedVac evaporator.

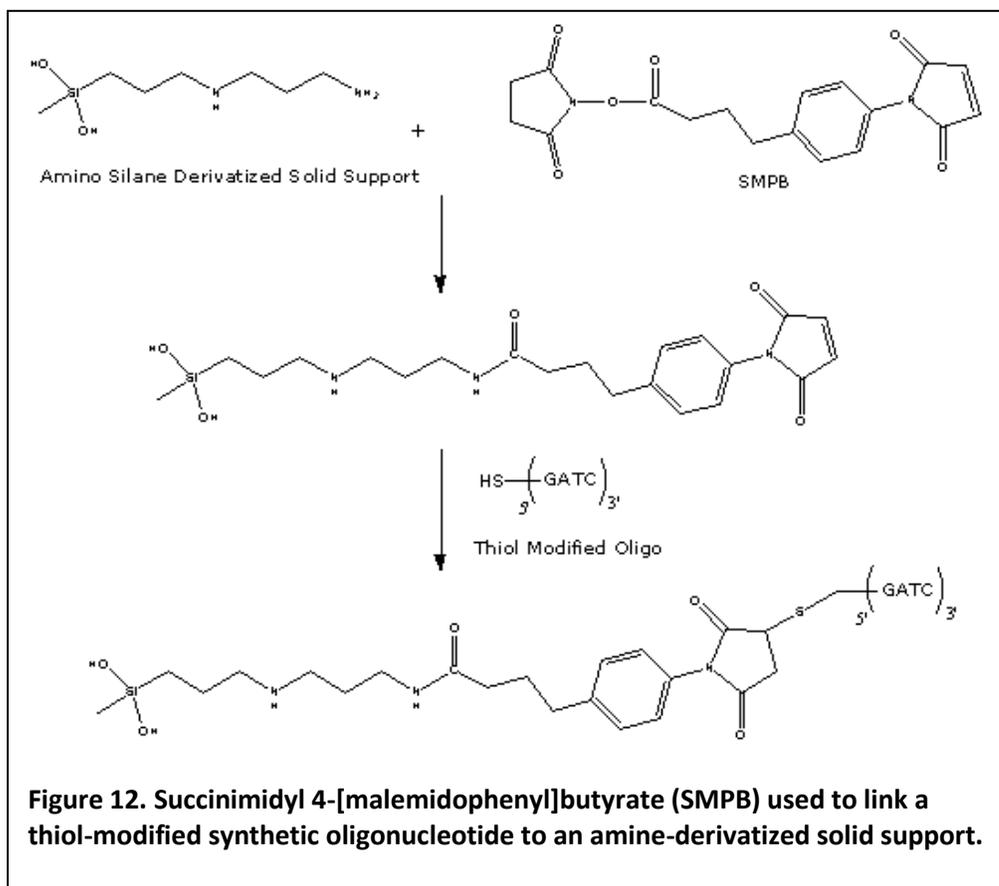
Any oligonucleotide that is not used immediately should be stored frozen. Over time, the oligo will oxidize and the above procedure will need to be repeated before coupling.

4.3.4 Attaching Thiol Modified Oligonucleotides

As a class, the cross-linkers used to attach thiol-modified oligonucleotides to solid supports are heterobifunctional, meaning that they possess functional groups capable of reaction with two chemically distinct functional groups, e.g. amines and thiols. The linkers serve two purposes: to covalently bind two distinct chemical entities which otherwise would remain un-reactive toward each other and as a physical spacer which provides greater accessibility and/or freedom to each of the linked biomolecules [35].

A number of heterobifunctional cross linkers have been developed for covalent attachment of thiol-modified DNA oligomers to aminosilane monolayer films [35]. These cross linkers combine groups reactive toward amines such as N-hydroxy-succinimidyl esters and groups reactive toward thiols such as maleimide or alpha-haloacetyl moieties. Figure 12 illustrates the use of one such cross linker, succinimidyl 4-[maleimidophenyl]butyrate (SMPB) to link a thiol-modified oligo to an amine derivatized solid support.

The use of SMPB to cross link thiol modified oligos and aminosilanized glass slides has resulted in a surface density of approximately 20 pmoles of bound DNA/cm² [35]. This density was significantly greater than that reported for DNA films formed on similar substrates prepared using aminosilane films with a diisothiocyanate cross linker [1] or epoxysilane films [5].



4.4 Acrydite™-Modified Oligonucleotides

Proprietary small molecule attachment chemistry, developed by Apogent Discoveries (no longer in business), enables covalent attachment of macromolecules to surfaces via acrylic linkages. A macromolecule derivatized with Acrydite group can polymerize with free acrylic acid monomer to form polyacrylamide or can react with SH or silane surfaces. This chemistry will also work with plastic and other polymer surfaces.

An acrylic acid group can be directly attached to the 5'-end of an oligo nucleotide (with a 6-carbon linker arm) at the time of synthesis using Acrydite, an acrylic-phosphoramidite developed by Mosaic Technologies. The structure of 5'-acrydite modified DNA is shown in Figure 13.

The Acrydite-acrylamide group can be used to immobilize oligonucleotides, which are fully available for hybridization when bound to the solid support. Since the acrylamide group is thermostable, products derived from Acrydite-modified oligonucleotides, such as PCR fragments, can also be immobilized using the same chemistries (Figure 14).

4.4.1 Advantages of Acrydite chemistry

1. The Acrydite-modified material is stable *prior* to coupling. Acrydite-modified oligonucleotides are stable in aqueous solutions over a wide range of temperature and pH.
2. The Acrydite-modified material forms covalent bonds to solid phases. Attachment of the Acrydite-modified material to solid phases occurs with formation of highly stable carbon-

carbon or thioether bonds.

These bonds are stable in all conditions routinely encountered in standard molecular biology protocols.

3. Conjugation is efficient. Acrydite-modified DNA generally couples with greater than 90% efficiency.
4. Acrydite is versatile. Acrydite-modified DNA can be immobilized on glass, polymer or chromatography media.

- Glass microscope slides for microarrays. The well-known reaction of thiol groups with acrylamide forms the basis for a robust chemistry for attaching Acrydite-modified oligonucleotides to glass microscope slides for microarrays [39].

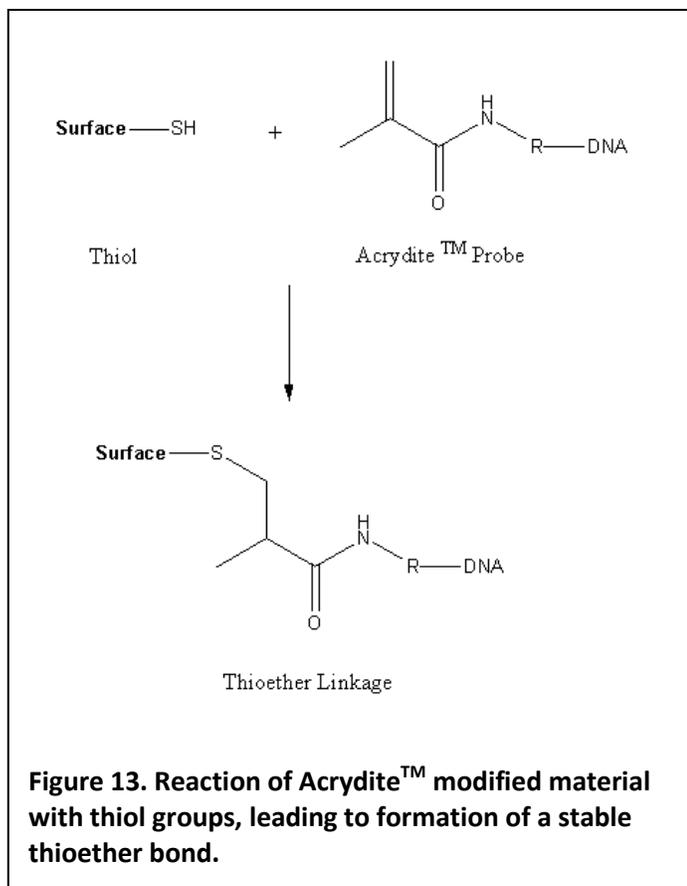


Figure 13. Reaction of Acrydite™ modified material with thiol groups, leading to formation of a stable thioether bond.

- When an aqueous solution Acrydite-modified material is copolymerized with another monomer such as acrylamide, and with a crosslinker such as bis, the resulting product is a crosslinked gel with pendant nucleic acids. Since free radical polymerization is a highly efficient process, the concentration of nucleic acids in the polymer is determined by the input concentrations, and the ratio of Acrydite-modified material to monomer.
- When the Acrydite-modified material is copolymerized with another monomer in the presence of an acryl coated surface (such as an acryl-silane coated plastic), the Acrydite material will copolymerize with the monomer to form a gel layer on the surface. The concentration of bound Acrydite and the properties of the gel layer can easily be adjusted over a wide range, so that the surface can be prepared with specific properties such as hydrophobicity.
- When the Acrydite-modified material is copolymerized with another monomer such as acrylamide, without crosslinker, the resulting product is a linear polymer with pendant nucleic acids. Linear polymers can be used in a wide variety of applications. For instance, linear polymers formed with some acrylamide derivatives are soluble at room temperature, but condense and precipitate above 45°C. This property of temperature dependent solubility can be used for various separation processes.

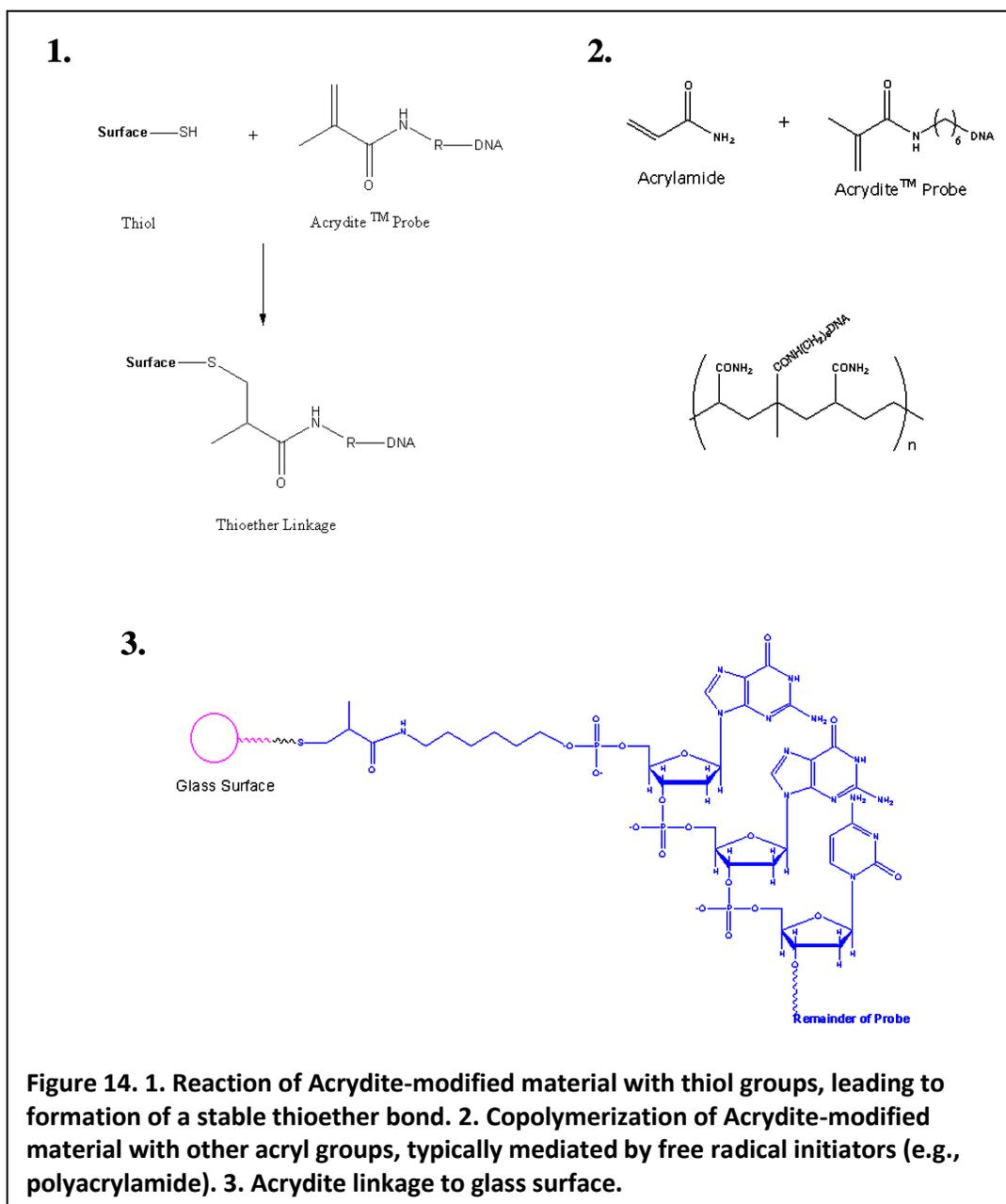
4.4.2 Acrydite Oligos from IDT

1. Available as a 5' modification only.

2. Available in 100 nmole, 250 nmole, 1 μ mole, and 10 μ mole scale reactions.
3. Does not require any special purification.
4. Available in 96-well plates.
5. Inquire for bulk discounts.

4.4.3 Attaching Acrydite-Modified Oligonucleotides

Proprietary small molecule attachment chemistry has been developed by Mosaic Technologies Inc. that enables covalent attachment of macromolecules to surfaces via acrylic linkages [39]. The Acrydite-acrylamide group has been used to immobilize oligonucleotides, fully available for hybridization, to thiol-modified glass slides. Since the acrylamide group is thermostable, products derived from Acrydite-modified oligonucleotides, such as PCR fragments, can also be immobilized using the same chemistries. Immobilization can be accomplished with standard inexpensive gel polymerization techniques that are already widely used in molecular biology laboratories. Immobilization does not require highly reactive and unstable chemical crosslinking agents. An Acrydite linkage can yield surface densities of ~ 200 fmole hybridizable probe/ mm^2 . This density compares favorably with literature values using other attachment methods [39].



5. Conclusion

Solid support attachment of oligonucleotides, including microarray technology, provides a powerful set of tools for molecular biologists. In order to insure that the data available from these applications are accurate, precise, and reliable, considerable effort in choosing and designing oligonucleotides is essential. Success begins with choosing the best method of attachment for the synthetic oligonucleotides. Oligonucleotides with amino, thiol, or Acrydite modifications have been the most reliable to date.

6. References

1. Guo Z, Guilfoyle RA, et al. (1994) Direct fluorescence analysis of genetic polymorphisms by hybridization with oligonucleotide arrays on glass supports. *Nucleic Acids Res*, 22(24): 5456–5465.
2. Kumar A, Larsson O, et al. (2000) Silanized nucleic acids: a general platform for DNA immobilization. *Nucleic Acids Res*, 28(14): E71.
3. Maskos U and Southern EM. (1992) Oligonucleotide hybridizations on glass supports: a novel linker for oligonucleotide synthesis and hybridization properties of oligonucleotides synthesised *in situ*. *Nucleic Acids Res*, 20(7): 1679–1684.
4. Lindroos K, Liljedahl U, et al. (2001) Minisequencing on oligonucleotide microarrays: comparison of immobilisation chemistries. *Nucleic Acids Res*, 29(13): E69–9.
5. Lamture JB, Beattie KL, et al. (1994) Direct detection of nucleic acid hybridization on the surface of a charge coupled device. *Nucleic Acids Res*, 22(11): 2121–2125.
6. Joos B, Kuster H, and Cone R. (1997) Covalent attachment of hybridizable oligonucleotides to glass supports. *Anal Biochem*, 247(1): 96–101.
7. Rogers YH, Jiang-Baucom P, et al. (1999) Immobilization of oligonucleotides onto a glass support via disulfide bonds: A method for preparation of DNA microarrays. *Anal Biochem*, 266(1): 23–30.
8. Mirzabekov AD. (1994) DNA sequencing by hybridization--a megasequencing method and a diagnostic tool? *Trends Biotechnol*, 12(1): 27–32.
9. Proudnikov D, Timofeev E, and Mirzabekov A. (1998) Immobilization of DNA in polyacrylamide gel for the manufacture of DNA and DNA-oligonucleotide microchips. *Anal Biochem*, 259(1): 34–41.
10. Beier M and Hoheisel JD. (1999) Versatile derivatisation of solid support media for covalent bonding on DNA-microchips. *Nucleic Acids Res*, 27(9): 1970–1977.
11. Arshady R. (1993) Microspheres for biomedical applications: preparation of reactive and labelled microspheres. *Biomaterials*, 14(1): 5–15.
12. Skowronski EW, Armstrong N, et al. (2000) Magnetic, microplate-format plasmid isolation protocol for high-yield, sequencing-grade DNA. *Biotechniques*, 29(4): 786–788, 790, 792.
13. Engelstein M, Aldredge TJ, et al. (1998) An efficient, automatable template preparation for high throughput sequencing. *Microb Comp Genomics*, 3(4): 237–241.
14. Yang L, Tran DK, and Wang X. (2001) BADGE, Beads Array for the Detection of Gene Expression, a high-throughput diagnostic bioassay. *Genome Res*, 11(11): 1888–1898.
15. Defoort JP, Martin M, et al. (2000) Simultaneous detection of multiplex-amplified human immunodeficiency virus type 1 RNA, hepatitis C virus RNA, and hepatitis B virus DNA using a flow cytometer microsphere-based hybridization assay. *J Clin Microbiol*, 38(3): 1066–1071.
16. Iannone MA, Taylor JD, et al. (2000) Multiplexed single nucleotide polymorphism genotyping by oligonucleotide ligation and flow cytometry. *Cytometry*, 39(2): 131–140.
17. Chen J, Iannone MA, et al. (2000) A microsphere-based assay for multiplexed single nucleotide polymorphism analysis using single base chain extension. *Genome Res*, 10(4): 549–557.

18. Cai H, White PS, et al. (2000) Flow cytometry-based minisequencing: a new platform for high-throughput single-nucleotide polymorphism scoring. *Genomics*, 66(2): 135–143.
19. Ye F, Li MS, et al. (2001) Fluorescent microsphere-based readout technology for multiplexed human single nucleotide polymorphism analysis and bacterial identification. *Hum Mutat*, 17(4): 305–316.
20. Armstrong B, Stewart M, and Mazumder A. (2000) Suspension arrays for high throughput, multiplexed single nucleotide polymorphism genotyping. *Cytometry*, 40(2): 102–108.
21. Connolly BA and Rider P. (1985) Chemical synthesis of oligonucleotides containing a free sulphhydryl group and subsequent attachment of thiol specific probes. *Nucleic Acids Res*, 13(12): 4485–4502.
22. Zuckermann R, Corey D, and Schultz P. (1987) Efficient methods for attachment of thiol specific probes to the 3'-ends of synthetic oligodeoxyribonucleotides. *Nucleic Acids Res*, 15(13): 5305–5321.
23. Sproat BS, Beijer B, et al. (1987) The synthesis of protected 5'-mercapto-2',5'-dideoxyribonucleoside-3'-O-phosphoramidites; uses of 5'-mercapto-oligodeoxyribonucleotides. *Nucleic Acids Res*, 15(12): 4837–4848.
24. Li P, Medon PP, et al. (1987) Enzyme-linked synthetic oligonucleotide probes: non-radioactive detection of enterotoxigenic *Escherichia coli* in faecal specimens. *Nucleic Acids Res*, 15(13): 5275–5287.
25. Dreyer GB and Dervan PB. (1985) Sequence-specific cleavage of single-stranded DNA: oligodeoxynucleotide-EDTA X Fe(II). *Proc Natl Acad Sci U S A*, 82(4): 968–972.
26. Nelson PS, Sherman-Gold R, and Leon R. (1989) A new and versatile reagent for incorporating multiple primary aliphatic amines into synthetic oligonucleotides. *Nucleic Acids Res*, 17(18): 7179–7186.
27. Nelson PS, Kent M, and Muthini S. (1992) Oligonucleotide labeling methods. 3. Direct labeling of oligonucleotides employing a novel, non-nucleosidic, 2-aminobutyl-1,3-propanediol backbone. *Nucleic Acids Res*, 20(23): 6253–6259.
28. Beattie WG, Meng L, et al. (1995) Hybridization of DNA targets to glass-tethered oligonucleotide probes. *Mol Biotechnol*, 4(3): 213–225.
29. Adessi C, Matton G, et al. (2000) Solid phase DNA amplification: characterisation of primer attachment and amplification mechanisms. *Nucleic Acids Res*, 28(20): E87.
30. Ghosh SS and Musso GF. (1987) Covalent attachment of oligonucleotides to solid supports. *Nucleic Acids Res*, 15(13): 5353–5372.
31. Wolf SF, Haines L, et al. (1987) Rapid hybridization kinetics of DNA attached to submicron latex particles. *Nucleic Acids Res*, 15(7): 2911–2926.
32. Lund V, Schmid R, et al. (1988) Assessment of methods for covalent binding of nucleic acids to magnetic beads, Dynabeads, and the characteristics of the bound nucleic acids in hybridization reactions. *Nucleic Acids Res*, 16(22): 10861–10880.
33. Gingeras TR, Kwok DY, and Davis GR. (1987) Hybridization properties of immobilized nucleic acids. *Nucleic Acids Res*, 15(13): 5373–5390.
34. Rasmussen SR, Larsen MR, and Rasmussen SE. (1991) Covalent immobilization of DNA onto polystyrene microwells: the molecules are only bound at the 5' end. *Anal Biochem*, 198(1): 138–142.

35. Chrisey LA, Lee GU, and O'Ferrall CE. (1996) [Covalent attachment of synthetic DNA to self-assembled monolayer films](#). *Nucleic Acids Res*, 24(15): 3031–3039.
36. O'Donnell MJ, Tang K, et al. (1997) [High-Density, Covalent Attachment of DNA to Silicon Wafers for Analysis by MALDI-TOF Mass Spectrometry](#). *Anal. Chem.*, 69(13): 2438–2443.
37. Bischoff R, Coull JM, and Regnier FE. (1987) [Introduction of 5'-terminal functional groups into synthetic oligonucleotides for selective immobilization](#). *Anal Biochem*, 164(2): 336–344.
38. Sinha ND and Cook RM. (1988) The preparation and application of functionalized synthetic oligonucleotides: III. Use of H-phosphonate derivatives of protected amino-hexanol and mercapto-propanol or -hexanol. *Nucleic Acids Res*, 16(6): 2659–2669.
39. Rehman FN, Audeh M, et al. (1999) [Immobilization of acrylamide-modified oligonucleotides by co-polymerization](#). *Nucleic Acids Res*, 27(2): 649–655.