# Custom Oligonucleotide Synthesis

Custom Oligo Synthesis, antisense oligos, RNA oligos, chimeric oligos,
Fluorescent dye labeled oligos, Molecular Beacons, siRNA, phosphonates
Affinity Ligands, 2'-5' linked Oligos

## Custom Amidite, NHS & Solid Support Specifications

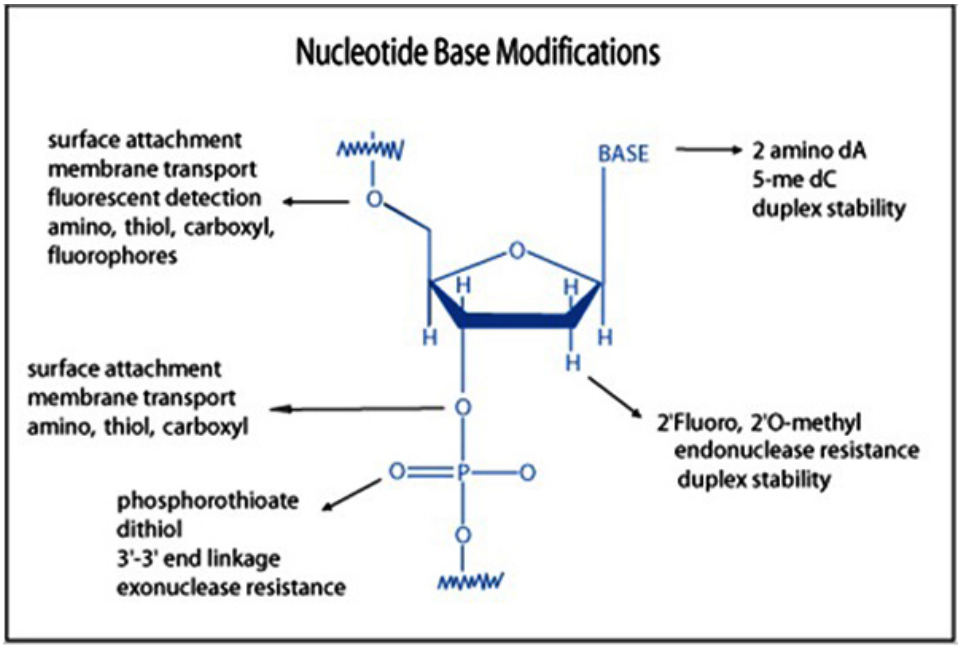
Custom Oligo Synthesis & Labelling

For Research Use Only. Not for use in diagnostic procedures for clinical purposes

30 years
results you can rely on
Gene Link™

# Custom Novel Modifications

On occasion researchers have developed special modified bases or simply have ideas of modified bases that they wish to be incorporated in oligos. Gene Link routinely collaborates extensively with investigators. We assist in the design of oligos with special modification to impart specific characteristics. We also incorporate investigator supplied modified bases and fluorescent dyes in oligos. If it is simply an idea, then we collaborate with other labs and companies for the synthesis of these novel modified bases. This guide summarizes the specifications and requirements. Contact us for more information at support@genelink.com



# Custom Amidite, NHS-modification & Solid Support Specifications

## Custom Beta-Cyanoethyl Phosphoramidite (CEP, Amidite)

At Gene Link for our entire standard DNA synthesis we use ß-cyanoethyl phosphoramidite (CEP, amidite) monomers; this being the industry standard. With this method, high coupling efficiencies are easily attained. Almost all modifications are also CEP.

Gene Link also on customer request would accept special modified CEP and incorporate this modification for custom oligo synthesis.

We require knowledge of the following that are related to using these as custom modifications in our automated instruments; we do not need to know any proprietary structure and use and thus do not divulge any confidential information.

1. Solubility in acetonitrile or inform us the solvent composition. We require 100% solubility at 0.1M concentration.
2. Should be free of particulate material.
3. Greater than 99% coupling efficiency at 0.1M concentration with a 3 minute coupling cycle.
4. Should have DMT (dimethoxy trityl) at 5' hydroxyl position.
5. Should have cyanoethyl phosphoramidite at 3' position.
6. Coupling should be amenable to standard DNA synthesis chemistry with deblocking, activation, capping and oxidation reagents. Inform us if it requires some other reagents.
7. Deprotection with 30% ammonium hydroxide or inform us of special deprotection protocol.
8. We require a minimum quantity of 100 µmole for initial quality control and coupling test. This quantity is also sufficient for up to 4 more couplings at the 1 µmolar scale of synthesis.

## Amidite Requirements

The table below details the quantity of amidite required for incorporation of the custom amidite. The usage per site is based on the Expedite DNA synthesizers with special protocol for incorporation of modified bases. The requirement listed below also takes into account test synthesis, priming and wastage.

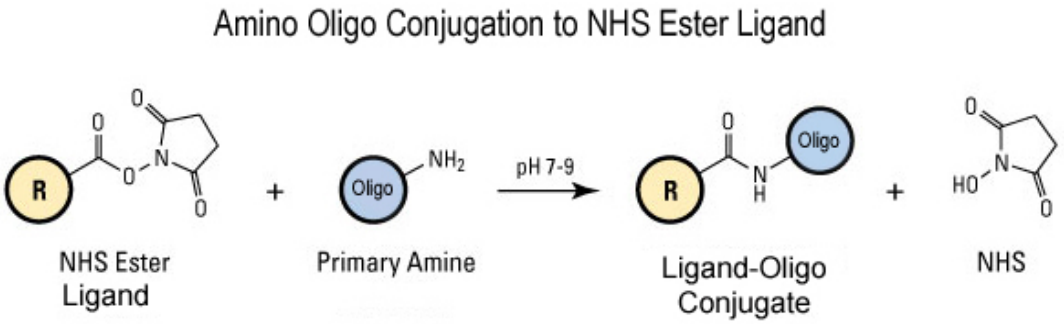
| Oligo Size & Scale of Synthesis | Quantity Required Per Site | Minimum Requirement | Purified Oligo Yield nmols & [mg] |
|---|---|---|---|
| Up to 50mer at 200 nmol scale | 12.5 µmol | 100 µmol | ~8 nmols [~0.15 mg] |
| Up to 130mer at 1 µmol scale | 20 µmol | 100 µmol | ~4 nmols [~0.25 mg] |
| Up to 250mer at 2 µmol scale | 40 µmol | 100 µmol | ~1 nmol [~0.15 mg] |

| Quantity Required Per Site | Quantity Required | Minimum Requirement |
|---|---|---|
| 1 | 12.5 µmol | 100 µmol |
| 10 | 200 µmol | 250 µmol |
| 20 | 400 µmol | 500 µmol |

## NHS-Modifications (*N*-Hydroxysuccinimide, N-succinimidyl ester, SE, NHS)

Generally, we prefer custom oligo modifications to be synthesized as a ß-cyanoethyl phosphoramidite (CEP, amidite). It is realized that not all modifications are easily amenable to be converted to the CEP form. The CEP form enables incorporation using automated DNA synthesis instruments and special protocols.

Numerous modifications are not available as CEP and we incorporate these using post-synthesis conjugation. Gene Link recommends NHS as the active functional group for conjugation to primary amino group incorporated into the oligos as shown below. Usually 2 mg of the NHS modification is adequate for one conjugation. The NHS-modification should be soluble in DMSO or DMF and free of any particulate matter.




## Custom Solid Support

We also accept custom solid supports to be utilized for oligo synthesis. The solid support should have the following specifications.

1. The modified base is attached to a solid support, preferably CPG. Other solid supports also accepted are polystyrene and nylon membrane.
2. The solid support should be of a pore size amenable for synthesis of oligo size. Refer to DNA synthesis chemistry background or contact Gene Link technical support.
3. The 5'-hydroxyl should have a DMT or appropriately attached to another group if it is not a base. DMT group is essential for use as a solid support.
4. The solid support linkage to the modified base should be cleaved by 30% ammonia with 90 minutes.
5. A minimum quantity of 100 mg is required for initial quality control and coupling test. This quantity is also sufficient for up to 2 more couplings at the 1 µmolar scale of synthesis.

### Product Description, Catalog Numbers & Code

| Product Description | Code* | Catalog Number |
|---|---|---|
| Custom DNA CEP (amidite) | [CDX] | 26-6591 |
| Custom DNA Conjugation (NHS) | [CDN] | 26-6592 |
| Custom DNA Support (CPG) | [CDS] | 26-6593 |
| | | |
| Custom RNA CEP (amidite) | [CRX] | 27-6591 |
| Custom RNA Conjugation (NHS) | [CRN] | 27-6592 |
| Custom RNA Support (CPG) | [CRS] | 27-6593 |

*Use the above codes in your oligo sequence at the specific site

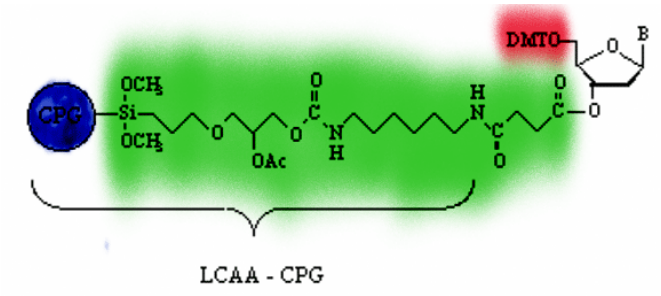
# Standard DNA Synthesis Chemistry Background

## Beta-Cyanoethyl Phosphoramidite Chemistry

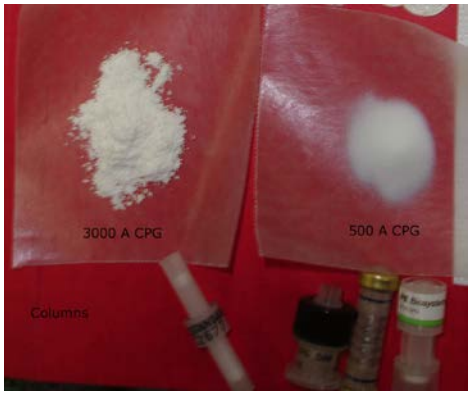
At Gene Link for our entire standard DNA synthesis we use ß-cyanoethyl phosphoramidite monomers; this being the industry standard. With this method, high coupling efficiencies are easily attained. The absence of side reactions also confers high biological activity of the synthetic oligonucleotide. In the basic reaction cycle, a solid support, derivatized with the initial protected nucleoside, is contained in a reaction column. Reagents and solvents are pumped through the column to couple the addition of successive protected nucleotide monomers (phosphoramidites, amidites). Each addition cycle includes detritylation, activation, coupling, oxidation, and capping. Intervening wash steps remove excess reactants and by-products of reaction. After the chain elongation is complete, the oligomer must be removed from the support and fully deprotected. The crude product is desalted and can be purified by reverse phase cartridge, polyacrylamide gel electrophoresis, or by HPLC.

## Solid Support

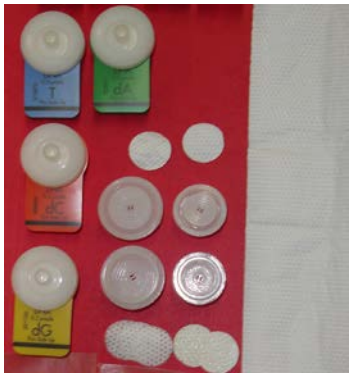
The synthesis of an oligomer begins with the selection of the reaction column containing the initial support-bound protected nucleoside. The reactive 5'-hydroxyl group of the support-bound nucleoside is protected with a dimethoxytrityl (DMT) group. The 3'-hydroxyl group of the nucleoside is covalently attached, through an appropriate hydrocarbon spacer, to the Controlled Pore Glass (CPG) support or other support of choice e.g polystyrene or nylon membrane. 500A CPG is recommended for oligomers up to 50 bases in length; 1000A CPG is recommended for oligomers more than 50 bases in length. Following this guideline provides better coupling efficiency by minimizing steric hindrance.




3' Base attached to CPG solid support



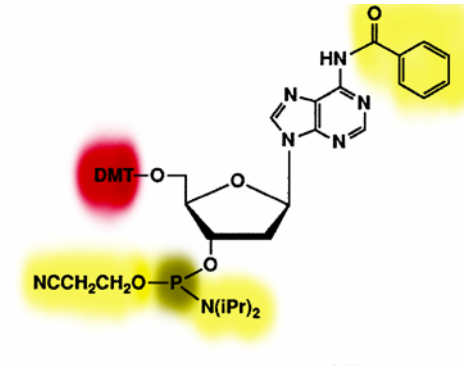

Controlled Pore Glass Solid Support



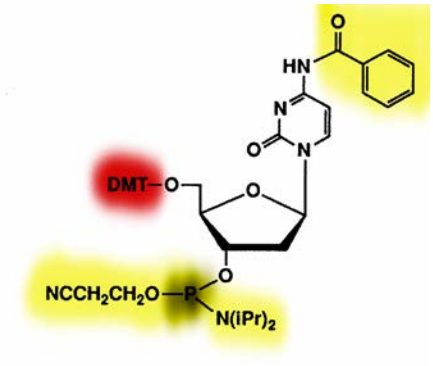
PVDF Membrane Solid Support

## Monomers

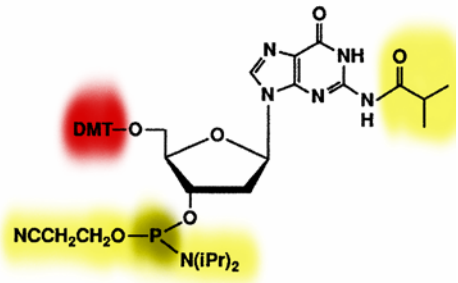
The exocyclic amines of 2'-deoxyadenosine (dA), 2'-deoxycytidine (dC) and 2' deoxyguanosine (dG) have to be protected during synthesis to prevent side reactions from taking place at these sites. The dC and dA monomers are protected by benzoyl (bz) groups at the N4 and N6 positions, respectively, while the dG is protected at the N2 position by an isobutyryl (ibu) group. These groups are removed after synthesis of the oligomer is complete, during deprotection. The phosphoramidite monomers are also protected at the the 5'-hydroxyl position with a DMT group, while the 3'-phosphite is modified by ß-cyanoethyl and diisopropylamine groups.



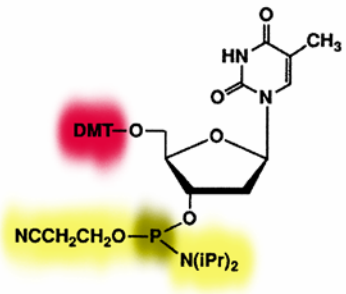

*N-Benzoyl 5'-O-DMT-2'-deoxy-Adenosine-3'-(β-cyanoethyl)-Phosphoramidite*




*N⁴-Benzoyl-5'-O-DMT-2'-deoxy-Cytidine-3'-(β-cyanoethyl)-Phosphoramidite*




*N-Isobutyrl-5'-O-DMT-2'-deoxy-Guanosine-3'-(β-cyanoethyl)-Phosphoramidite*




*5'-O-DMT-Thymidine-3'-(β-cyanoethyl)-Phosphoramidite*

## Detritylation

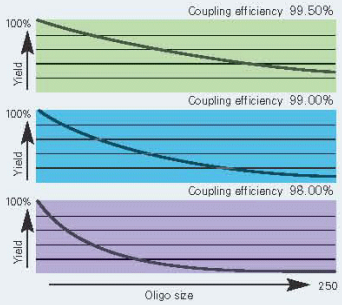
In the first step of the synthesis cycle, the acid labile DMT group of the support-bound monomer is removed with a dichloroacetic acid solution (DCA). The resulting cation is orange. The yield of the DMT cation can be estimated spectrophotometrically and be used to determine stepwise coupling efficiency. As the DNA bases are acid-labile, the detritylation step must only be as long as is necessary to ensure complete detritylation.

## Coupling

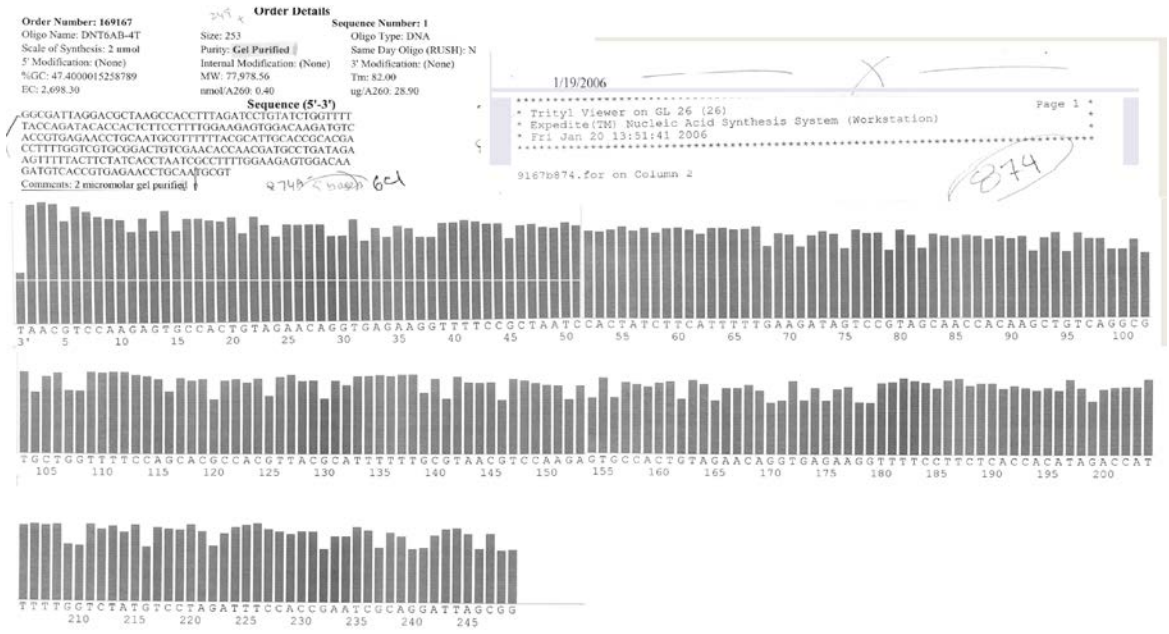
After detritylation the next protected phosphoramidite is delivered to the reaction column. Tetrazole is used to activate the phosphoramidite. The two reagents are mixed just prior to delivery to the reaction column. Tetrazole is a weak acid and it protonates the tertiary nitrogen group of the phosphoramidite so that the diisopropylamine moiety becomes a good leaving group.
The Coupling Mechanism is a nucleophilic attack by the free 5'-hydroxyl group on the 3'-phosphorous of the incoming activated monomer. For this reason, it is important to have a totally hydroxyl-free environment in the column. To ensure this, dry acetonitrile is used as the general solvent, and all the reagents and solvents are maintained in the anhydrous state. Under these conditions the coupling efficiencies are very high, thereby permitting synthesis of long oligomers.

**Coupling Efficiency and Full Length Oligo Yield**

| Oligo Size | 99.50% | 99.00% | 98.00% |
|---|---|---|---|
| 20 | 90.916 | 82.617 | 68.123 |
| 40 | 82.243 | 67.573 | 45.48 |
| 60 | 74.398 | 55.268 | 30.363 |
| 80 | 67.301 | 45.204 | 20.27 |
| 100 | 60.881 | 36.973 | 13.533 |
| 120 | 55.074 | 30.24 | 9.034 |
| 140 | 49.821 | 24.734 | 6.031 |
| 160 | 45.068 | 20.23 | 4.027 |
| 180 | 40.769 | 16.546 | 2.688 |
| 200 | 36.88 | 13.533 | 1.795 |
| 220 | 33.36 | 11.07 | 1.19 |
| 240 | 30.18 | 9.05 | 0.8 |
| 250 | 28.7 | 8.19 | 0.65 |




PCR and sequencing reactions are very robust and can tolerate up to 50% failure/truncated sequence oligos. However, you are clearly taking a chance by using long oligos synthesized at anything below 99.5% coupling efficiency.




**Trityl bars Coupling efficiency of each base addition**

## Oxidation

The most recently added monomer is now linked to the chain by trivalent phosphite bond. However, phosphorus linkages are more stable when the oxidation state is pentavalent, as is the case in native DNA. Therefore, the bond is oxidized in an iodine solution.

## Capping

Since 1-2% of the free 5'-hydroxyl groups do not undergo reaction, unreacted chains (failure sequences) must be capped to prevent further elongation in the next cycles. For this step, acidic anhydride and N-methylimidazole are mixed to form an activated acetylating agent.

## Cycling

Following the capping step, the cycle of reactions is repeated, beginning with the detritylation step, until the chain elongation is complete.

## Final Detritylation

If the oligomer is to be purified by Oligo-Pak column methods or by Reverse Phase HPLC, the DMT group is left on the 5'-OH of the oligomer and is removed only after purification. If the oligomer is to be purified by gel electrophoresis or ion exchange HPLC, the oligonucleotide is detritylated at this stage.

## Removal from Support and Deprotection

After the specified sequence has been synthesized, the oligo must be removed (cleaved) from the support and fully deprotected prior to use.

A 90 minute room temperature treatment with ammonium hydroxide is used to cleave the oligomer from the support and to deprotect the phosphorus by ß-elimination of the cyanoethyl group. A 24-hour room temperature treatment or an 8 hour 55°C treatment with ammonium hydroxide effectively removes the capping groups and the benzoyl and isobutyl groups protecting the exocyclic amines.

After cleavage/deprotection, the resulting crude mixture contains the tritylated product oligomer, the truncated failure sequences with free 5'-hydroxyl ends, by-products of deprotection (benzamide, isobutyramide, acrylonitrile, and acetamide), and silicates from hydrolysis of the glass support.

## Purification

The crude oligonucleotide can be purified using reverse phase chromatography or by gel electrophoresis. Reverse phase chromatography using manual cartridges or HPLC depend on the hydrophobic trityl group to separate the product from the failure sequences. Reverse phase purification is not recommended for oligos longer than 40mer as single strand oligos themselves are hydrophobic. All Gene Link oligos shorter than 40mer usually does not require any further purification if the application is for PCR or sequencing. Gel purification is strongly advised for all applications involving cloning of the product, example mutagenesis, cloning or gene construction application.

## References

Agrawal, S. (1993). Protocols for Oligonucleotides and Analogs, 20. Agrawal S. (1994). Protocols for Oligonucleotide Conjugates, 26. Beaucage, S. L. and Caruthers, M. H. (1981). Tetrahedron Lett., 22, 1859. Eckstein, F. (1991). Oligonucleotides and Analogies. Letsinger, R. L. finnan, J. L., Heavner, G. A., and Lunsford, W. B. (1975). J. Am. Chem. Soc., 97, 3278. McBride, L. J. and Caruthers, M. H. (1983). Tetrahedron Lett., 24, 245. Matteucci, M. D. and Caruthers, M. H. (1980). Tetrahedron Lett. 21, 719.

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