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

Gene Link, Inc. 190 Saw Mill River Road, Hawthorne, NY 10532, USA | www.genelink.com | support@genelink.com
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 β-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.

<table>
<thead>
<tr>
<th>Oligo Size &amp; Scale of Synthesis</th>
<th>Quantity Required Per Site</th>
<th>Minimum Requirement</th>
<th>Purified Oligo Yield nmols &amp; [mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up to 50mer at 200 nmol scale</td>
<td>12.5 mg</td>
<td>100 mg</td>
<td>~8 nmols (~0.15 mg)</td>
</tr>
<tr>
<td>Up to 130mer at 1 µmol scale</td>
<td>20 mg</td>
<td>100 mg</td>
<td>~4 nmols (~0.25 mg)</td>
</tr>
<tr>
<td>Up to 250mer at 2 µmol scale</td>
<td>40 mg</td>
<td>100 mg</td>
<td>~1 nmol (~0.15 mg)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Quantity Required Per Site</th>
<th>Quantity Required</th>
<th>Minimum Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20 mg</td>
<td>100 mg</td>
</tr>
<tr>
<td>10</td>
<td>200 mg</td>
<td>250 mg</td>
</tr>
<tr>
<td>20</td>
<td>400 mg</td>
<td>500 mg</td>
</tr>
</tbody>
</table>
NHS-Modifications (N-Hydroxysuccinimide, N-succinimidyl ester, SE, NHS)

Generally we prefer custom oligo modifications to be synthesized as a \( \beta \)-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. Generally 2 mg of the NHS modification is sufficient 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.
Standard DNA Synthesis Chemistry Background

Beta-Cyanoethyl Phosphoramidite Chemistry
At Gene Link for our entire standard DNA synthesis we use β-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 effect 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.
**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 5'-hydroxyl position with a DMT group, while the 3'-phosphite is modified by β-cyanoethyl and diisopropylamine groups.

**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 step-wise 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, a weak acid, protonates the tertiary nitrogen group of the phosphoramidite so that the diisopropylamine moiety becomes a good leaving group.

The Coupling Mechanism is a nucleophylic 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

<table>
<thead>
<tr>
<th>Oligo Size</th>
<th>99.90%</th>
<th>99.80%</th>
<th>99.00%</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>80.61%</td>
<td>28.10%</td>
<td>08.23%</td>
</tr>
<tr>
<td>40</td>
<td>82.20%</td>
<td>67.57%</td>
<td>45.48%</td>
</tr>
<tr>
<td>60</td>
<td>84.18%</td>
<td>65.26%</td>
<td>30.30%</td>
</tr>
<tr>
<td>80</td>
<td>77.38%</td>
<td>45.10%</td>
<td>20.27%</td>
</tr>
<tr>
<td>100</td>
<td>61.21%</td>
<td>36.91%</td>
<td>13.53%</td>
</tr>
<tr>
<td>120</td>
<td>56.74%</td>
<td>30.64%</td>
<td>9.04%</td>
</tr>
<tr>
<td>140</td>
<td>49.01%</td>
<td>24.72%</td>
<td>6.11%</td>
</tr>
<tr>
<td>160</td>
<td>45.06%</td>
<td>20.14%</td>
<td>4.02%</td>
</tr>
<tr>
<td>180</td>
<td>40.70%</td>
<td>15.45%</td>
<td>2.08%</td>
</tr>
<tr>
<td>200</td>
<td>36.86%</td>
<td>13.33%</td>
<td>1.76%</td>
</tr>
<tr>
<td>220</td>
<td>33.90%</td>
<td>11.07%</td>
<td>1.19%</td>
</tr>
<tr>
<td>240</td>
<td>30.18%</td>
<td>9.06%</td>
<td>0.75%</td>
</tr>
<tr>
<td>250</td>
<td>28.70%</td>
<td>8.19%</td>
<td>0.85%</td>
</tr>
</tbody>
</table>

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.
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 assembled, the oligomer 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 β-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 isobutyryl 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**

Document Warranty and Liability

Information in this document is subject to change without notice. This document and all information presented in this document are written as a guide. Gene Link, Inc. does not warrant this document to be free of errors and assumes no responsibility for any errors that may appear in this document. Gene Link disclaims all warranties with respect to this document, expressed or implied, including but not limited to those of merchantability or fitness for a particular purpose. In no event shall Gene Link be liable, whether in contract, tort, warranty, or under any statute or on any other basis for special, incidental, indirect, punitive, multiple or consequential damages in connection with or arising from this document, including but not limited to the use thereof.

Website

As the receipt of information on the Internet is highly dependent upon factors, including without limitations to, the user’s computer, browser, operation system, etc., information may be perceived incorrectly. Therefore, Gene Link does not warrant or guarantee that the information contained on its website www.genelink.com is error free.

Product Warranty and Liability

Warranty: Gene Link makes no warranty of any kind, specifically disclaims and excludes all other warranties of any kind or nature, directly or indirectly, express or implied, including, without limitation, as to the suitability, productivity, durability, fitness for a particular purpose or use, merchantability, condition, or any other matter with respect to Gene Link products. Gene Link products are for research purposes only including custom products. There is no warranty or claim of its performance for any specific research application. All Gene Link products are guaranteed to meet or exceed the specifications stated. Each Gene Link product is shipped with documentation stating specifications and other technical information. If the product fails to meet the stated specifications the sole remedy is prompt replacement by Gene Link or within 30 days of purchase a refund of the purchased price.

Liability. Under no circumstances shall Gene Link be liable for any damages directly or indirectly related to Gene Link’s products and services. Whether direct, incidental, foreseeable, consequential, or special (including but not limited to loss of use, revenue or profit), whether based upon warranty, contract, tort (including negligence) or strict liability arising in connection with the sale or the failure of Gene Link products to perform in accordance with the stated specifications.

Research Use Only. Not for use in diagnostic or clinical procedures.

Notice to Purchaser: The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser’s activities for a fee or other form of consideration. For information on obtaining additional rights, please contact support@genelink.com.

© 2017 Gene Link Inc. All rights reserved.
The trademarks mentioned herein are the property of their respective owners.

Gene Link, Inc.
190 Saw Mill River Road
Hawthorne, NY 10532
USA

Tel: (914) 769-1192
Email: support@genelink.com
www.genelink.com