Oligonucleotide Synthesis

Oligonucleotide synthesis (or gene synthesis) is used for several applications in medicine and life science research:

- Primers for DNA sequencing and amplification (PCR)
- Probes for detecting complementary DNA or RNA via hybridization
- Synthesis of artificial genes
- Anti-sense oligonucleotides (several siRNAs in clinical development)
- Tools for targeted introduction of genetic mutations

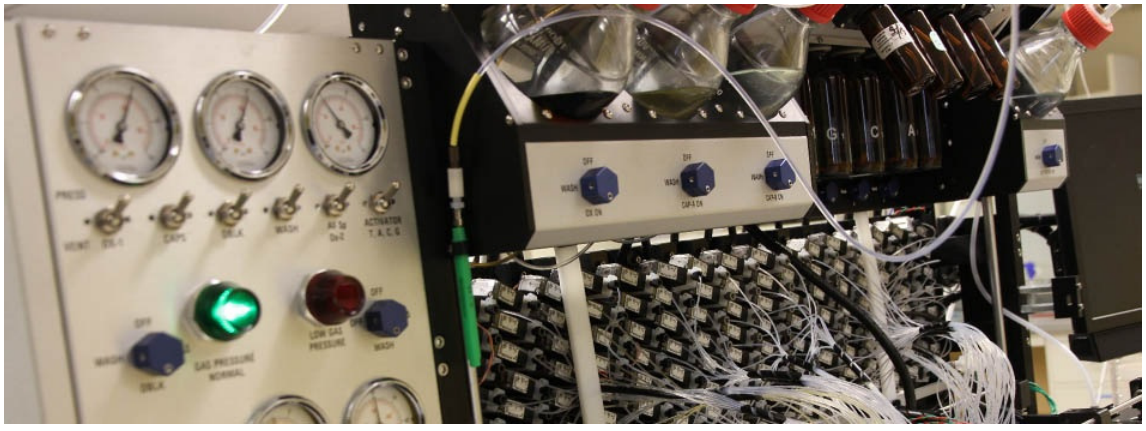
Trends in oligonucleotide synthesis:

- Prior to automated methods of oligo production, oligonucleotide synthesis was typically carried out manually in solution or on solid phase supports using glass columns equipped with porous filters (1).
- Today, oligonucleotide synthesis is carried out **automatically** on solid-phase supports using computer-controlled instruments (column and multi-well format)
    - The column format is ideally suited for research and large scale applications where a high-throughput is not required (2)
    - Multi-well plate format is designed specifically for high-throughput synthesis on small scale (3)
- A number of oligonucleotide synthesizers for small scale synthesis and medium to large scale synthesis are available commercially.

DNA synthesis market (over 400 qualified accounts globally):

- Custom primer businesses
    - Integrated DNA technologies, Life Technologies, Operon, Eurofins, etc.
- Pharmaceutical & biotech companies creating siRNA, or anti-sense therapeutics
    - Pilot scale, early-scale R&D
- Academic core labs (study of gene mutation and creation of stable cell lines)

Oligosynthesizers:

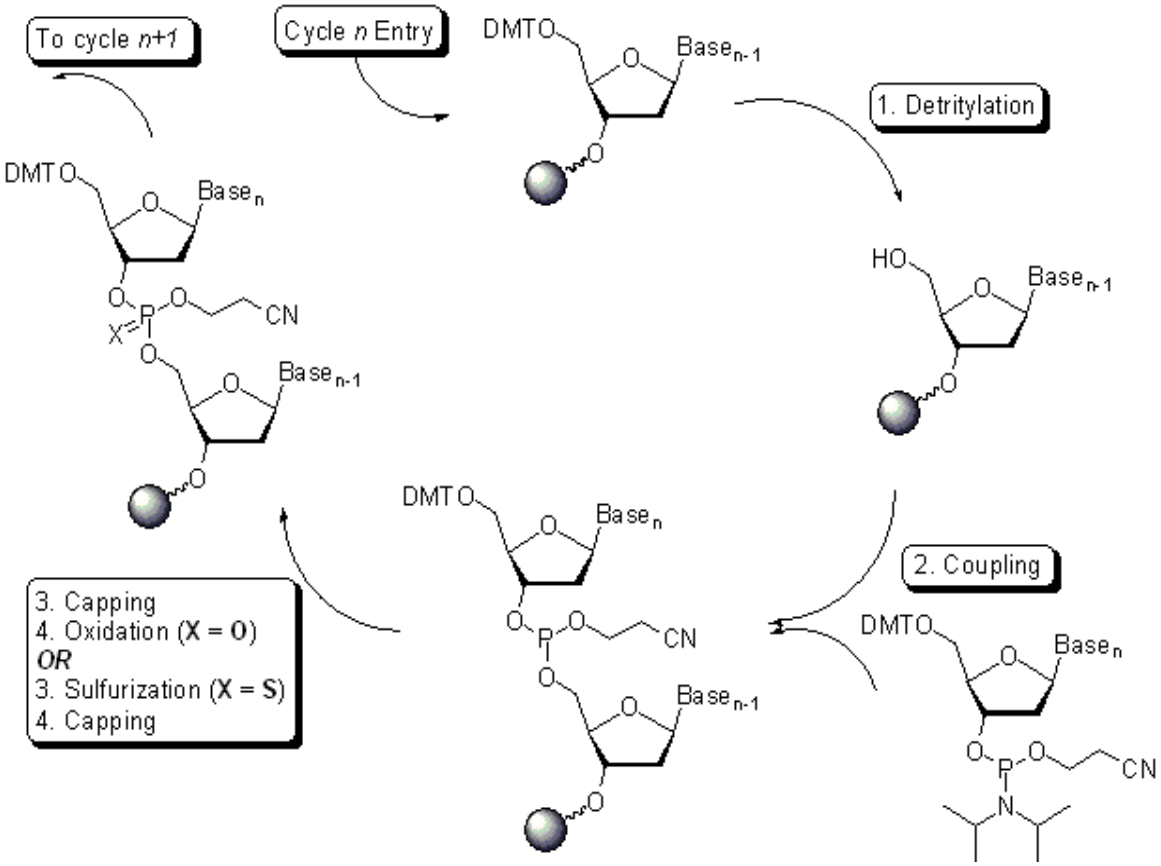


Typical requirements and blends used by oligo synthesis customers:

Oligosynthesis Reagents	Blend/Description	Typical Product Specs
Acetonitrile Low Water	Acetonitrile ≤ 30 ppm water	Water ≤ 30 ppm
		Acidity ≤ 0.5 µE/g
		Purity (GC) min. 99.9%
Acetonitrile Anhydrous	Acetonitrile Anhydrous ≤ 10 ppm water	Water ≤ 10 ppm
		Acidity ≤ 0.5 µE/g
		Purity (GC) min. 99.9%
Activator Solution (ETT)	0.3M ETT in Acetonitrile	Assay, ETT = 0.28 – 0.32M
		Water ≤ 50 ppm
		HPLC purity 99.8% min.
Activator Solution (BTT)	0.25 M BTT in Acetonitrile	Assay, BTT = 0.24 – 0.26 M
		Water ≤ 30 ppm
		HPLC purity 99.5 % min.
Activator Solution (DCI)	0.25 M DCI in Acetonitrile	29-32 g/L (Dicyanoimidazole, titration)
		≤30 ppm water content (KF)
		0.25 M Concentration
Deblock DCM Solution	3% TCA in Dichloromethane	Assay, TCA = 30g/l ± 3%
		2.7 – 3.3% (w/v)
		Water ≤ 150 ppm
Deblock DCE Solution	3% TCA in Dichloroethane	Assay, TCA = 30g/l ± 3%
		2.7 – 3.3% (w/v)
		Water ≤ 100 ppm
Dichloromethane Low Water		Water ≤ 30 ppm
		Acidity ≤ 0.1 µEq/g
Cap A, 10% Pyridine	THF/Pyridine/Ac2O (8:1:1)	Ac_2O = 9.0 – 11.0% (v/v)
		10.1 - 11.9% (w/v)
		Pyridine = 9.0 – 11.0% (v/v)
		8.8 – 10.8% (w/v)
Cap A, Mild	Tetrahydrofurane / TAC2O 100/5 (v/w)	45-55 g/L gravimetric (TAC2O)
Cap A, 10% Ac2O in THF	Acetic Anhydride / Tetrahydrofurane 9.1/90.9 (v/v)	88.7-108.4 g/L (Acetic Anhydride content, titration)
Cap B, 16% NMI in THF	THF/1-Methylimidazole (84:16)	NMI = 15.2 – 17.8% (v/v)
		15.7 – 18.3% (w/v)
		Water ≤ 120 ppm
Cap B, 10% Pyridine	Tetrahydrofurane / N-Methylimidazole / Pyridine 8/1/1 (v/v/v)	≤200 ppm (water content, Karl Fischer)
Oxidizer, 20% Pyridine	0.02M Iodine in THF/Pyridine/Water (70:20:10)	Iod = 20mM ± 10%
		Pyridine = 18.5 – 21.5%
		Release Date ≤ 6 month
Oxidizer, 10% Pyridine	0.02M Iodine in THF/Pyridine/Water (70:10:20)	Iod = 4.8-5.5 g/L
		Pyridine = 9.5 – 10.5%
		THF = 69.5 - 70.5%
Oxidizer, Low Pyridine	Tetrahyrofuran/Water/Pyridine/Iodine 90.54/9.05/0.41/0.43 (v/v/v/w)	0.02 M iodine
		90.54/9.05/0.41/0.43 (v/v/v/w)

Oligonuleotide synthetic cycle:

- Oligonucleotide synthesis is carried out by a stepwise addition of nucleotide residues to the 5'-terminus of the growing chain until the desired sequence is assembled. Oligonucleotide synthesis uses modified nucleotides called phosphoramidites. Each addition is referred to as a synthetic cycle and consists of four chemical reactions (18):



Automated oligonucleotide synthesis and GC capabilities overview:

	Oligonucleotide Synthesis Step	**GC typically offers in:**
Step 1	***Deblocking (de-tritylation)*** • Deblock Solution: Tricholoroacetic Acid or Dichloroacetic Acid in dichloromethane or toluene	4L Amber Glass
Step 2	***Activation*** • *1-H-tetrazole dicyanoimidazole (DCI) or ethylthio-1H-tetrazole (ETT) in acetonitrile*	*Catalog versions of components*
Step 3	***Coupling*** • Anhydrous Acetonitrile • Activators	Acetonitrile in Drums/Bottles
Step 4	***Capping*** • Cap A: Acetic anhydride, pyridine and THF • Cap B: n-methylimidazole in THF	Cap A/Cap B in Drums/Bottles
Step 5	***Oxidation*** • Blend: Iodine in THF, pyridine and water	4L Bottles
Step 6	***Post synthesis: Deprotection & Desalting*** • Chromatography solvents • Chemicals for SDS PAGE	Drums/Bottles

Step 1: De-blocking (detritylation)

The DMT groups on phosphoramidites are first removed with an acid solution, such as **2% trichloroacetic acid (TCA)** or **3% dichloroacetic acid (DCA)**, in an inert solvent (typically **dichloromethane** or **toluene**). The DMT cations that are formed are then washed out and the result is a solid support-bound oligonucleotide precursor bearing a free 5'-terminal hydroxyl group.

Step 2: Activation

Nucleoside phosphoramidite mixtures (up to 0.2M) in **acetonitrile** are first activated by a 0.2–0.7 M solution of an acidic azole catalyst **(i.e., 1*H*-tetrazole, 2-ethylthiotetrazole** (4)**, 2-benzylthiotetrazole** (5,6)**,** or **4,5-dicyanoimidazole** (7)). The mixing is typically brief and oftentimes occurs in the fluid lines of oligonucleotide synthesizers.

Step 3: Coupling

The activated phosphoramidites (up to 20-fold excess over the support-bound oligonucleotides) are then exposed to either the starting solid support or to a support-bound oligonucleotide precursor. The 5'-hydroxy group reacts with the activated phosphoramidite

moiety of the incoming nucleoside phosphoramidite to form a phosphite triester linkage (8,9,10). Note: The reaction is highly sensitive to water. For this reason, oligonucleotide manufacturers carry out coupling reactions in **anhydrous acetonitrile**.

Upon the completion of the coupling reactions, any unbound reagents and by-products are then removed by washing.

Step 4: Capping

The capping step is performed by treating the solid support-bound material with a mixture of **acetic anhydride** and **1-methylimidazole** (or, less often, **DMAP**)

- After the coupling reaction, a small amount of the solid support-bound 5'-OH groups (0.1 to 1%) remains unreacted and needs to be permanently blocked from further chain elongation to prevent the formation of (n-1) shortmers, which have an internal base deletion. These unreacted 5'-hydroxy groups are acetylated by the capping mixture.

Step 5: Oxidation

The newly formed phosphite triester linkages are not natural and possess limited stability. The treatment of the support-bound material with iodine and water in the presence of a weak base (**pyridine, lutidine,** or **collidine**) oxidizes the phosphite triester into a stonger, tetra-coordinated phosphate triester. The result is a protected precursor of the naturally occurring phosphate diester internucleosidic linkage. Oxidation may also be carried out under anhydrous conditions using **tert-Butyl hydroperoxide** (11) or, more efficiently, (1S)-(+)-(10-camphorsulfonyl)-oxaziridine (CSO) (12,13,14).

Step 6: Post-synthesis

After the completion of the chain assembly, the solid support-bound oligonucleotide is fully protected. To produce a functional oligonucleotide however, all the protecting groups have to be removed by treatment with inorganic bases or amines.

- Note: This method is limited because acrylonitrile is created as a side product. The formation of this side product may be avoided by treating the solid support-bound oligonucleotides with solutions of bases in an organic solvent, such as 50% **triethylamine** in **acetonitrile** (15) or 10% **diethylamine** in **acetonitrile** (16). This treatment is strongly recommended for medium- and large scale preparations.

Solid support-bound oligonucleotides are deprotected with aqueous **ammonium hydroxide, aqueous methylamine** (17), or a mixture of the two.

The fully deprotected product is then purified by a number of methods (where Fisher Chemical chromatography solvents are used).

- **Most commonly, the crude product is desalted using ethanol precipitation, size exclusion chromatography, or reverse-phase HPLC**
- **To eliminate unwanted (n-1) shortmers, the oligonucleotides can be purified via polyacrylamide gel electrophoresis**
- **Unwanted by-products can also be purified by anion-exchange HPLC followed by desalting**

References:

1. Tanaka, Toshiki; Letsinger, R. L. (1982). "Syringe method for stepwise chemical synthesis of oligonucleotides". *Nucl. Acids Res.* **10** (10): 3249–3259. doi:10.1093/nar/10.10.3249.
2. "OligoMaster LS2". Azcobiotech.com. http://www.azcobiotech.com/instruments/OligoMaster.php. Retrieved 2011-10-18.
3. "DNA / RNA Oligonucleotide Synthesizer: MerMade 384". Bioautomation.com. http://www.bioautomation.com/Synthesizers-MerMade%20384.html. Retrieved 2011-10-18.
4. Sproat, B.; Colonna, F.; Mullah, B.; Tsou, D.; Andrus, A.; Hampel, A.; Vinayak, R. (1995). "An efficient method for the isolation and purification of oligoribonucleotides". *Nucleosides & Nucleotides* **14** (1&2): 255–273. doi:10.1080/15257779508014668.
5. Stutz, A.; Hobartner, C.; Pitsch, S. (2000). "Novel fluoride-labile nucleobase-protecting groups for the synthesis of 3'(2')-O-amino-acylated RNA sequences". *Helv. Chim. Acta* **83** (9): 2477–2503. doi:10.1002/1522-2675(20000906).
6. Welz, R.; Muller, S. (2002). "5-(Benzylmercapto)-1H-tetrazole as activator for 2'-O-TBDMS phosphoramidite building blocks in RNA synthesis". *Tetrahedron Lett.* **43** (5): 795–797. doi:10.1016/S0040-4039(01)02274-2.
7. Vargeese, C.; Carter, J.; Yegge, J.; Krivjansky, S.; Settle, A.; Kropp, E.; Peterson, K.; Pieken, W. (1998). "Efficient activation of nucleoside phosphoramidites with 4,5-dicyanoimidazole during oligonucleotide synthesis". *Nucl. Acids Res.* **26** (4): 1046–1050. doi:10.1093/nar/26.4.1046. PMC 147346. PMID 9461466.
8. Ogilvie, K. K.; Usman, N.; Nicoghosian, K.; Cedergren, R. J. (1988). "Total chemical synthesis of a 77-nucleotide-long RNA sequence having methionine-acceptance activity". *Proc. Natl. Acad. Sci. USA* **85** (16): 5764–5768. doi:10.1073/pnas.85.16.5764. PMC 281845.
9. Wu, T.; Ogilvie, K. K.; Perreault, J. Pierre; Cedergren, R. J. (1989). "Convenient procedure for the preparation of specific mixed DNA-RNA polymers". *J. Amer. Chem. Soc.* **111** (22): 8531–8533. doi:10.1021/ja00204a043.
10. Pon, R. T. (1987). "Enhanced coupling efficiency using 4-dimethylaminopyridine (DMAP) and either tetrazole, 5-(o-nitrophenyl)tetrazole, or 5-(p-nitrophenyl)tetrazole in the solid phase synthesis of oligoribonucleotides by the phosphoramidite procedure". *Tetrahedron Lett.* **28** (32): 3643–3646. doi:10.1016/S0040-4039(00)96344-5.
11. Alul, R. H.; Singman, C. N.; Zhang, G.; Letsinger, R. L. (1991). "Oxalyl-CPG: a labile support for synthesis of sensitive oligonucleotide derivatives". *Nucleic Acids Res.* **19** (7): 1527–1532. doi:10.1093/nar/19.7.1527.
12. "New Product: 0.5M CSO for non-aqueous oxidation in DNA synthesis". Glenres.com. http://www.glenresearch.com/GlenReports/GR22-23.html. Retrieved 2013-01-28.
13. ^ Manoharan, M.; Lu, Y.; Casper, M. D.; Just, G. (2000). "Allyl Group as a Protecting Group for Internucleotide Phosphate and Thiophosphate Linkages in Oligonucleotide Synthesis: Facile Oxidation and Deprotection Conditions". *Org. Lett.* **2** (3): 243–246. doi:10.1021/ol9910518.
14. Prakash, T. P.; Johnston, J. F.; Graham, M. J.; Condon, T. P.; Manoharan, M. (2004). "2'-O-[2-[(N,N-dimethylamino)oxy]ethyl]-modified oligonucleotides inhibit expression of mRNA in vitro and in vivo". *Nucleic Acids Res.* **32** (2): 828–833. doi:10.1093/nar/gkh220.
15. Capaldi, D. C.; Gaus, H.; Krotz, A. H.; *et al.* (2003). "Synthesis of High-Quality Antisense Drugs. Addition of Acrylonitrile to Phosphorothioate Oligonucleotides: Adduct Characterization and Avoidance". *Org. Proc. Res. & Development* **7** (6): 832–838. doi:10.1021/op020090n.
16. Volume 5: Deprotect to completion in organic solvents. *Glen Report* **22** (2)

17. Boal, J. H.; Wilk, A.; Harindranath, N.; Max, E. E.; Kempel, T.; Beaucage, S. L. "Cleavage of oligodeoxyribonucleotides from controlled-pore glass supports and their rapid deprotection by gaseous amines". *Nucl. Acids Res.* **24** (15): 3115. PMC 146024. PMID 8760903.
18. http://en.wikipedia.org/wiki/File:Oligocycle1.png