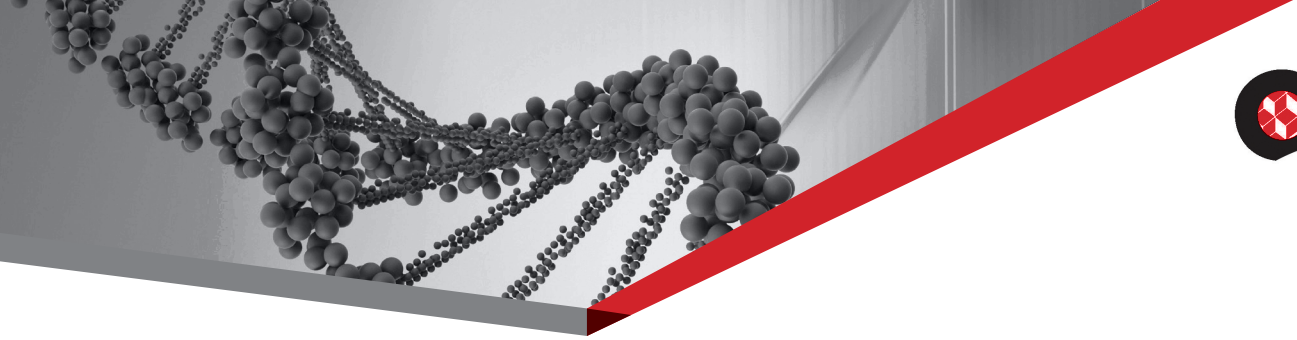

OLIGO FACTORY

Methods for Purification of Synthetic Oligonucleotides

Why is purification required in the manufacturing of synthetic oligonucleotides?

With the refinement of synthesis chemistry and automation, synthetic oligonucleotides are readily available. Complex oligonucleotide sequences can be assembled with reasonable affordability and ease. Currently almost all oligonucleotides sequences are assembled on an automated instrument using a solid support either pre-derivatized with a nucleoside or with a universal linker.

In order to assemble the sequence, addition of each nucleoside requires four chemical reaction steps and each of these reaction steps is expected to be quantitative (100%). In reality, efficiencies of these reaction steps vary from 97 to 99.9% depending on the reagents, nucleoside building blocks (phosphoramidite) and moisture in the solvent. Depending on the efficiency of each nucleoside addition (four reaction steps), oligonucleotide sequences of twenty nucleosides (20-mer) and twenty five nucleosides (25-mer), theoretically would result in the following percentage of desired oligonucleotide (as shown in the table).

Stepwise Coupling Efficiency	% of full length oligo (20-nucleosides)	% of full length oligo (25-nucleosides)
97.0%	57.8	49.6
98.0%	68.2	61.6
98.5%	87.3	69.6
99.0%	82.6	78.6

Even though precautions are taken to prevent side reactions, the sensitive nature of nucleosides often result in undesired side reactions including depurination, branching and modification of nucleosides, which at the end of synthesis results in a mixture of oligonucleotides. The mixture consist of several sequences, including shorter sequences: described as n-1, n-2, n-3, n-4, etc; longer sequences, described as n+1, n+2, etc. (due to over coupling), and finally modified sequences. In automated oligonucleotides synthesis, the product is not purified after each nucleoside addition, which is in contrast to multi step small molecule synthesis where the product is purified/enriched after each reaction step. Hence, when the side chain protecting groups are removed, a mixture of oligonucleotides is released from the solid support along with benzamide & isobutyramide (the side chain protecting groups). The desired full-length oligonucleotide is typically the main component (depending on coupling efficiency and sequence length) in the mixture. The deprotected oligonucleotides can be isolated and purified by several different methods outlined below. The method of choice will depend on the applications and purity required.

Precipitation: It is one of the simplest methods of isolation. In this method, benzamide, isobutyramide and very smaller sequences (di-, tri-, tetra-, penta- mers) are removed from deprotected oligonucleotides. Precipitated material thus obtained is suitable for sequencing, PCR primer and cloning.

Gel Filtration or Size-Exclusion Gel Filtration Isolation: Gel Filtration is a separation method based on the molecules size. The stationary phases (gel media such as Sephadex G-25 or Sephadex G-50) are materials with well defined range of pore sizes. Smaller oligonucleotides pass through inside the pores and therefore take a longer time to elute. Longer oligonucleotides are excluded from entering the pores, and hence are eluted in the void volume and therefore are eluted first. Sephadex G-25 and Sephadex G-50 can remove shorter oligonucleotides from 25-mers and 50-mers respectively. This method can be also used for desalting purposes. Purity of the isolated material may vary depending on the quality of synthesis. The resultant material is suitable for PCR, sequencing or cloning.

Reverse Phase Cartridge Purification: The less complicated RP cartridge/column purification is based on the hydrophobic interaction of oligonucleotides [containing 5'-O-Dimethoxytrityl (DMT) or modified with hydrophilic groups such as dyes, cholesterol, disulfide linker, tocopherol, etc.] with lipophilic C-18 purification media. The cartridges are packed with C-18 silica or polystyrene based C-18. Oligonucleotides with a hydrophobic group are retained tightly by the C-18 media present in the cartridge. Oligonucleotides without hydrophilic groups are retained less tightly and easily washed off with water and a low percentage of acetonitrile. The tightly retained oligonucleotides are isolated with a higher percentage of acetonitrile. In the case of 5'-O-DMT containing oligonucleotides, the 5'-O-DMT-group is removed with acid treatment (1 min exposure to 2% TFA solution for removal of DMT) after purification. This method results in a fairly pure material (~90%) but is unable to remove shorter sequences containing 5'-O-DMT- group or N+ mers. Resulting material is also suitable for sequencing, cloning, PCR or preliminary screening purposes.

Preparative PAGE (Poly acrylamide gel electrophoresis) method: Oligonucleotides with negative charge migrate on the basis of mass to charge ratio through the pores of polyacrylamidie gel during electrophoresis, generally polyacrylamidie containing urea (7M) which denatures the oligonucleotides. The composition of polyacrylamide gel varies from 30-10% depending on the length of oligonucleotides to be purified. Migrated oligonucleotides separate as a band and can be visualized by UV shadowing under fluorescent background. The desired oligonucleotide (full length product) is the slowest migrating band. The band is excised, crushed and suspended in water and product isolated by gel filtration free from urea and migrating buffer. The disadvantage of this method is only very small amounts of oligo can be purified at a time and the yield is quite poor. The biggest advantage of this method is it results in an oligo with purity greater than 95%. The oligo, thus purified by this method are often used for X-ray crystallography, gene synthesis, and mutagenesis studies.

HPLC purification methods: Since the late 1980s, oligonucleotides are being explored for therapeutic and diagnostic purposes. These applications often require gram to kilogram quantities of oligonucleotides with high purity. These amounts are beyond the capabilities (and capacities) of the methods described above. HPLC (high pressure liquid chromatography) purification provides the opportunity to purify large amounts of oligonucleotide at high purity. Commonly two types of chromatography, Reverse Phase and Anion Exchange, are used for the purification of oligonucleotides.

(i) **Reverse Phase** Chromatographic Purification depends on the hydrophobic nature of the oligonucleotide. More hydrophobic oligos are retained stronger on the purification media than less hydrophobic materials, which can be easily removed by eluting with a lower percentage of organic solvent (acetonitrile or methanol/ethanol) in aqueous buffer. The more hydrophobic material is eluted with the higher percentage of organic solvent. Generally, C-18 silica or polystyrene based reverse phase media are used for purification using an aqueous buffer (0.1M TEAA / sodium acetate, pH =7) and an organic solvent (acetonitrile/ methanol) gradient. Sequences without a lypophilic group elute earlier than strongly hydrophobic material (as these materials have a strong hydrophobic interaction with the stationary phase). Synthetic oligonucleotides with the DMT (dimethoxytrityl)-group or modified oligonucleotides with dyes/biotin/ cholesterol (more hydrophobic) can be purified easily using this technique This process can easily be scaled up to produce hundreds of grams material.

(ii) **Anion Exchange** Chromatographic Purification depends on the interaction of the negatively charged oligonucleotides with quaternary ammonium ions or tertiary amine present on the stationary phase (purification media). Purification media functionalized with quaternary ammonium ions are described as strong anion exchangers (SAX) and media functionalized with tertiary amine are described as weak anion exchangers. Use of a mobile phase with increasing percentage of ionic concentration (or increasing molarity of the mobile phase) weakens the interaction of negatively charged oligonucleotides with the positively charged stationary phase. Shorter sequences with less negative charge elute with lower percentage of ionic (salt) solution. Oligonucleotides with non-complex structures are easily purified by this method. Oligodeoxynucleotide (DNA) sequences with higher percentage of guanosine or oligoribonucleotides (RNA) and modified RNA may possess more complex or secondary structures. These oligonucleotides, when purified, may result in multiple peaks on analysis by anion exchange HPLC, at neutral pH and ambient temperature. Therefore, purification of these oligonucleotides is also complicated due to secondary structures and purification can't be easily achieved by a simple SAX method at ambient temperature and neutral pH. Higher pH, higher temperature or chaotropic agents (salts, urea, organic solvent etc.) can destabilize these secondary structures and often result in a single peak. Purification of these types of oligonucleotides requires method development as higher pH or higher temperature may not be possible to use for every oligonucleotide. Purification of oligonucleotides containing deoxynucleosides and 2'-OMe nucleosides, can be performed at high pH (12) using a gradient of sodium chloride solution. Oligonucleotides containing ribonucleosides and 2'-F nucleosides are not stable at higher pH; hence purification of these can't be carried out with sodium chloride gradient at higher pH. Chaotropic salts (NaBr, NaClO4, LiCl or LiClO4) gradient or urea (2-7M) in NaCl solution gradient may be used for purification at ambient temperature. Lithium salts are very efficient chaotropic agent and may help in purification of these types of oligonucleotides but these salts may not be used for purification of therapeutic grade oligonucleotides. Presence of lithium salt may elicit a neurotoxicity response. Alternatively, purification can be performed at higher temperature by heating the HPLC column (60oC) and using a gradient of sodium chloride solution (60oC). The collected hot fractions require immediate cooling.

Desalting of the Purified Material: The material purified by either RP-HPLC or Anion Exchange-HPLC method contains free salt (excess) or buffer. In order to isolate the purified oligonucleotides, excess salt must be removed. One of the simplest methods of desalting is dialysis against distilled water, but this method is applicable only for small samples. Large scale desalting is performed by a bench top tangential flow filtration (TFF) system. The combined purified fractions are concentrated to a small volume and desalted by diafiltration with 7-12 exchanges of HPLC grade water resulting in conductivity of the solution to 30µS/cm or lower.

THE DECISION OF WHICH PURIFICATION METHOD TO USE IS DETERMINED BY THE NATURE OF THE OLIGONUCLEOTIDE SYNTHESIZED, THE AMOUNT TO BE PURIFIED, THE DEGREE OF PURIFICATION REQUIRED FOR THE APPLICATION, AND THE EQUIPMENT AVAILABLE FOR PURIFICATION. THE DECISION IS EMPIRICAL, I.E. EITHER BASED ON THE EXPERIENCE OF THE CHROMATOGRAPHER AND/OR THROUGH METHODS DEVELOPMENT BY AN EXPERIENCED CHROMATOGRAPHER. THE DECISION PROCESS IS NON-TRIVIAL AND REQUIRES THE JUDGEMENT OF EXPERIENCED AND INFORMED CHEMISTS AND CHROMATOGRAPHERS.