Oligonucleotide Purification, Concentration Determination & Stock Solution Preparation

Oligonucleotides (ONs) are synthesized by solid phase techniques on a column. Depending on your use, they can be ordered fully synthesized, but (a) still on the column with protecting groups in place, (b) cleaved from the column and deprotected. The former category is used primarily for situations where you plan to do post-synthetic modifications immediately on receiving the strands. For most purposes in our lab, we are using the latter category. The DNA received in the vials with this option will have free hydroxyl groups on the 3' and 5' end, or in the case of modifications, will have the modifier on either the 3' or the 5' end (discussed later).

Synthetic oligonucleotides, as a nature of the solid-phase synthetic process used, have truncated strands present corresponding to each base added. The addition of a base has a 99% yield, and the final step of the synthetic cycle "caps" any unreacted oligonucleotides, preventing them from reacting in the next addition cycle. This ensures that no "wrong" sequence oligonucleotides will be made, but the 1% unreacted oligonucleotide will remain. For most purposes, these truncated sequences are small enough contributions that they do not need to be purified from the main product. If it is necessary to remove them, HPLC (High Performance Liquid Chromatography) and PAGE (Poly-Acrylamide Gel Electrophoresis) are the methods of choice. Either step starts necessitates desalting (removing small molecules and salts) before more involved purification.

Desalting Oligonucleotides:

Following cleavage from the column, the oligonucleotides are dried. This process leaves residual small molecules from the deprotection, including high salt concentrations. To remove these small molecules, leaving only the longer ON strands, we use Sephadex G25 spin columns, which will remove small molecules and strands less than 10 bp. Sephadex is a proprietary cross-linked dextran gel that is frequently used for size exclusion columns. The following steps will walk you through purifying your ON with an Illustra Microspin G25 spin column.

Each spin column can hold up to 150 µL of solution, up to around 400 nmol of DNA. For the larger (1 µmol) synthesis batches, they must be split into multiple columns. For the smaller 200 nmol synthesis batches, one column is sufficient to purify each batch of DNA.

1. Re-suspend your dried ON with ultrapure water. Use 450 µL for the larger 1 µmol batches, and 150 µL for the smaller 200 nmol batches.
2. Vortex your vial to dissolve the sample. Mix with a pipette tip only if necessary, as this may cause sample loss to the pipette tip.
3. Prepare G-25 spin columns as indicated in the product literature.
    a. Briefly, each spin column is supplied as a suspension of the resin in water. The suspension must be even, requiring vortexing. Once the suspension is even, the water is centrifuged out, leaving behind a moist, packed column of the resin.
    b. Many centrifuges cannot be set to exactly 735 RCF. In this case, setting the speed lower than required can result in the column not fully forming, so the next higher speed should be chosen.
4. Carefully add 150 µL of crude ON to the top of the spin column, taking care not to touch the column with the pipette tip.
5. Place the column in a 1.7 mL centrifuge tube labeled with the ON name, and spin for 2 minutes at 735 RCF.
    a. For more complete collection, water can be added (100 µL) and the column spun a second time. This second fraction will be less pure than the first, but may contain additional ON. This fraction should be kept separately from the main fraction, and not combined.
    b. Many centrifuges cannot be set to exactly 735 RCF. A slower speed can result in incomplete collection of your product, while a higher speed can result in a higher proportion of smaller sequences/small molecules being passed through.

6. Keep the column in a tube, labeled, until you are sure that you no longer need any potential amounts of oligonucleotide that are stuck on it.
7. In the case of larger synthetic batches necessitating multiple runs, all first run fractions should be combined for concentration determination.

Calculating RCF for a Centrifuge:

The G-force (Relative Centrifugal Force) of a centrifuge is a factor both of the RPM it is set to, and the radius of rotation- longer radii will give higher forces at the same speed. The below equation can be used to calculate RFC from a given RPM:

$$RCF\ (G) = 1.12R\left(\frac{RPM}{1000}\right)^2$$

where *R* is the radius of rotation of the centrifuge, in millimeters. Different centrifuges will have different radii of rotation. Most can be found online or in the user manual for a specific centrifuge.

To check your work, you can use any of the readily available calculators online. More modern centrifuges have an internal conversion where RCF can be set directly.

Determining Concentration of Oligonucleotides:

As with any chemical species, the concentration of an oligonucleotide is proportional to its absorbance. The constant of proportionality is the **molar extinction coefficient**, frequently denoted by **ε**. The Beer-Lambert Law (frequently called Beer's Law) gives the equation used to calculate concentration (C, in M) from absorbance (A) at a given path-length, l.

$$A = \varepsilon lC$$

Molar Extinction Coefficients for DNA can be computed from the nearest-neighbor method. For lab today, you'll use IDT's OligoAnalyzer to approximate ε for each of your ONs. This program will incorporate 5'- and 3'- modifications as well as internal modifications in addition to your base sequence.

https://www.idtdna.com/calc/analyzer

Note the resulting extinction coefficients units (L/mol*cm), or $M^{-1}cm^{-1}$. The common pathlength for UV-Visible cuvettes is 1 cm.

Using a quartz cuvette, dilute 1-2 µL of your DNA stock into 1 mL of ultrapure water, and measure the absorbance using one of the Cary50 instruments. Use the absorbance, coupled with your dilution factor, to figure out the concentration of each of the ON stocks.

Sample Storage

For each of your sequences, you should record in your notebook:

1. Name & Sequence
2. Molecular Weight
3. Molar Extinction Coefficient

For structures of modifiers, Glen Research's catalogue is the source for all modified bases or phosphoramidites we use, and has the structures before addition to DNA that can be used to figure out the final structure of your sequence with any modifiers attached.

For storage, oligonucleotides will slowly degrade in water over time due to base-catalyzed hydrolysis of the phosphate ester bonds. Low temperature slows down hydrolysis, but freezing oligonucleotide solutions in water can promote shearing of strands on thawing. Additionally, storing all of your oligonucleotide stock in one tube makes it much more likely that contamination or sample loss will occur. Accordingly, it is ideal to store oligonucleotides in small aliquots dry at low temperatures.

Depending on the concentration of your oligonucleotide stock, it should be diluted to give a final concentration of 0.5 or 1 mM, which will make all subsequent calculations easier. The stock should then be divided into aliquots of 25 nmol DNA per vial.

If suitable instrumentation is available to dry your stocks, all but one vial should be dried to a solid and stored at -80°C. If no such instrumentation is available, the liquid aliquots should be stored at -20°C.

Drying Samples with a SpeedVac

The best way to store DNA (and RNA) samples is dry at -80°C (or -20°C if a low-temperature freezer is unavailable). Due to the high boiling point of solvent usually used for DNA solutions (i.e., water), drying samples without temperature induced degradation can be challenging. A SpeedVac is an instrument that uses a technique similar to rotatory evaporation to dry biological samples at lower temperatures under reduced pressure. For DNA, temperatures between 50°C and 60°C can be used with minimal degradation of the DNA to yield dry samples which can be safely stored for later use.

SpeedVac drying is also useful for increasing the concentration of a DNA sample- frequently, purification results in a lower than desired stock concentration. By drying a sample of a known amount of DNA, it can subsequently be rehydrated in a reduced volume, increasing the concentration of the stock.

A SpeedVac consists of two main components- a heated, pressure sealed centrifuge chamber and a pump system to maintain the reduced pressure. If a high-vacuum oil or belt driven pump is used, a cold trap between the chamber and pump is necessary to prevent any solvent removed from the DNA from condensing in the pump oil and causing corrosion.

For the SpeedVac in the Shared Instrument Room on the 1st floor, the following are the instructions for use:

1. Aliquot your DNA samples such that each centrifuge tube contains 25 nmol of DNA- this is an adequate amount for a number of successive studies, but limits the time it will be used such that little degradation occurs.
2. To open the top of the speedvac, lift up on the front and back of the glass and slide it backwards until it stops. At this point, the front can be lifted to rotate the slab back, allowing access to the rotor.
3. Balance your centrifuge tubes in the rotor, with lids open.
4. Close the top lid of the SpeedVac, and pull it back to its original position.
5. Turn on the pump.
6. Turn on the SpeedVac (green button).
7. Set the temperature dial to desired temperature between 50°C and 60 °C.
8. Turn on the SpeedVac heating element (orange button).
9. Turn on the Speedvac centrifuge (white button). After reaching speed, the vacuum should turn on and the chamber will seal. If the chamber does not seem to be sealing, gentle pressure applied to the top lid can help.
10. Drying time is dependent on the amount of liquid, number of samples, and whether or not the SpeedVac chamber was already warm. 50-100 µL samples of water should take 30 minutes to 1 hour to dry down. Check back occasionally to determine progress.
11. To check on or remove the samples, turn off the centrifuge (white button). When the rotor stops spinning and the vacuum seal has been released, open the chamber (as in step 2) to remove your samples.

12. Turn off the heating element, SpeedVac and pump.
13. Store samples in a cryo-safe box in the freezer until needed.