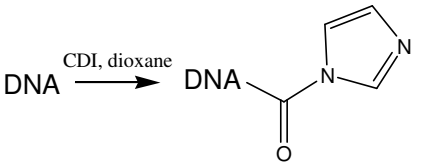
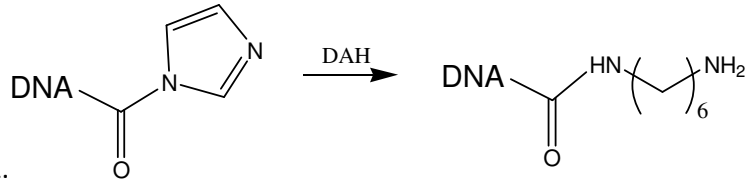
Conjugation of a thiol linker to synthetic DNA oligonucleotides

Kelley Laboratories, 2012

Part 1: Addition of amine linker:

1. 

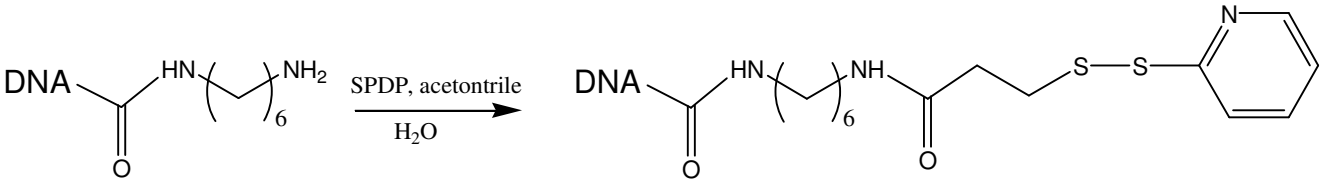
2. 

2.5 µmol synthesis of DNA (left on resin, trityl protecting group removed)
200 mg, 1, 1'-Carbonyl diimidazole (CDI) (Sigma), stored at 4°C in a desiccator
200 mg, 1,6 Hexamethylene diamine (DAH)(Sigma), stored at 4°C in a desiccator
1,4-dioxane, anhydrous (Aldrich), sureseal cap
Methanol
Balloon *(used to maintain anhydrous conditions when withdrawing dioxane)*
3 or 5 ml syringes
18 ga needles
2 dram glass vials
Ammonium hydroxide
Peptide shaker/coupling tube w/ rubber septum
0.45 (or 0.2) µm microcentrifuge filters (Costar)

1. Set up a peptide shaker in a fume hood with access to a vacuum and Argon gas.
2. Pour 1-2 ml dioxane in a glass vial. Cut off the filter end of the DNA tube with a sharp razor blade on top of a sheet of weighing paper. Pour beads into the glass vial of dioxane. *Save some beads at this step to deprotect along with the modified DNA. Beads are prone to static charge – removal of gloves and collection in dioxane reduces flyaways during this step.*
3. Pipette beads into the peptide coupling tube. Rinse the beads with 3 ml dioxane.
4. Dissolve 200 mg CDI in 2 ml dioxane. Seal off the top of the peptide coupling tube with a septum, and attach the argon line to the bottom of the tube. Using a 5 ml syringe, inject the CDI into the peptide coupling tube. *Ensure that the stopcock is closed.*
5. Turn on a very low flow of Argon, and open the stopcock. Vent the top of the coupling tube with a needle. Enough gentle bubbling should occur that the material is moving but the bubbles should not be vigorous or exceed 1 cm in height. *Hold the septum while starting the Ar flow because if too much pressure is allowed through the shaker, the septum with needle can be pushed off.*
6. Allow bubbling to continue for 30 min, then turn off the Ar flow and drain the liquid using vacuum. *This reaction can go upwards of 1 hr.*
7. Wash the resin 5 times with 3 ml of dioxane, shaking the beads vigorously and draining each time. Wash the beads from the sides of the tube on the final wash.
8. Dissolve 200 mg DAH in 2 ml dioxane and inject using the syringe into the peptide shaker. *Ensure that the stopcock is closed during this step.*

9. Bubble with Argon (see step 5) for 25 min.
10. Drain the liquid and wash 4 times with 3 ml of dioxane. Then, remove the septum and wash 3 times with 30 ml methanol.
11. Allow resin to dry by running the vacuum until no liquid is left on the resin or on the walls of the shaker (~5 min)
12. Collect the resin by pouring it out onto weighing paper and pour into a 2-dram glass vial with screw top.
13. Using a glass pipette, add 2-3 ml of ammonium hydroxide into the vial and incubate at 55°C overnight (no more than 16 hrs) in order to deprotect. *Deprotect the unmodified beads in the same manner.*
14. Take the vial containing the DNA/ammonium hydroxide and place it in the freezer to cool for a few minutes. Using a glass pipette, transfer the liquid to a spin filter (Costar, 0.2 µm nylon). Spin at max speed for 1 min. Collect the filtrate. Repeat until all the liquid is filtered.
15. Dry the DNA on a speed vac at 55-60°C.

Part 2: Addition of disulfide.



Amine-terminated DNA
Succiminidyl 3-(2-pyridyldithio)propionate (SPDP) (Sigma)
0.4 M HEPES (pH 8)
Anhydrous acetonitrile
Ammonium acetate (50 mM) (For HPLC)
Acetonitrile (For HPLC)
0.45 (or 0.2) µm microcentrifuge filters (costar)

1. Dissolve DNA in 100 µl 0.4 M HEPES. *Set aside a few µl for analytical HPLC. **<u>This solution can be kept at -20°C for up to 2 months.</u>*
2. Weigh out 4 mg of SPDP and put it into a microcentrifuge tube.
3. Add 25 µL acetonitrile (anhydrous) to dissolve the SPDP.
4. Add the SPDP solution to 50 µL DNA solution. *The reaction can be scaled up if desired.*
5. Secure the tube to the vortex using tape, and shake at low (setting 3) for one hour.
6. After one hour, add 75-100 µL H_2O to the reaction to precipitate out excess SPDP. Spin the vial at max speed for 1 min, then decant off the supernatant and filter it through a 0.2 µm microcentrifuge filter.
7. HPLC purify the DNA, collect in a 15 ml conical tube, freeze in LN_2 and lyophilize. *Cover with foil.*
   ***<u>The solid disulfide can be kept for up to 1 month at -20°C. Disulfide in solution can be kept for up to two weeks at -20°C.</u>*

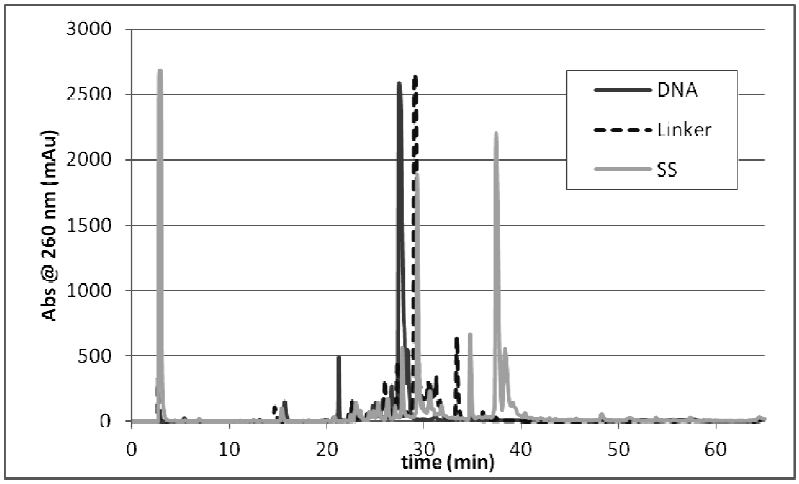
HPLC Suggestions:

Gradient: 0-20% acetonitrile vs 50 mM ammonium acetate over 60 min, 20-60% over 5 min. Total run length = 65 min. 5 min post-run.

Column:

Analyticals: Use a Microsorb-MV, C18, 100 Å, 250x4.6 mm column (Varian), flow rate 1.0 ml/min. Run the unmodified DNA sample and the DNA + linker samples to determine reaction yields at each step.

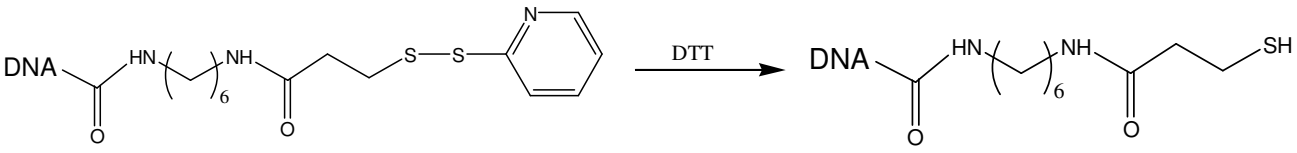
Preps: Use a reverse phase C18 column, 100 Å, 250x10.0 mm prep column, flow rate 3.5 ml/min.



Analytical chromatograms of a 20-mer of DNA (sequence DP32) at different points in the synthetic process. Run on a C18, 250x4.6 mm column, 1 ml/min flow rate. Elution times: Unmodifed DNA (27 min); Amine-terminated DNA (29 min); disulfide-modified (37 min).

The synthesis can be also monitored by electrospray ionization mass spectrometry (see Mass Spec notes).

Part 3: Conversion of disulfide to thiol:



Dithiothreitol, (DTT) electrophoresis grade (Bioshop)

100 mM sodium phosphate, pH 9

Nap-5 column (optional)

1. Resuspend the lyophilized DNA in 50 µl of water. *Or 50 mM phosphate buffer pH 8.*
2. Dissolve 15.4 mg DTT in 1 mL 100 mM NaPi (1 M solution). *Or 50 mM PB pH 8.*
3. Add 50 µl 1 M DTT to 50 µl DNA. Let sit at room temp for 30 min- 2 hrs. *Cover with foil.*
4. Purify the cleaved DNA via HPLC. Freeze in LN_2 and lyophilize to dryness.

Alternately: Prepare a Nap-5 column. Add 100 µL of the DNA DTT reaction to the column. When the liquid has entered the column, add 400 µL of H_2O to the column. Discard the eluent, and place the column over a clean microcentrifuge tube. Elute the DNA with 500 µL of H_2O. If higher concentrations are desired, collect 100 µl fractions and measure the absorbance at 260 to determine the fraction with the highest concentration.

Mass Spec Notes:

The identity of the collected HPLC fractions can be determined using electrospray ionization mass spectrometry (ESI-MS). Using 5 µL of water, pipet up and down to dissolve a small amount of the lyophilized powder to submit to MS.

Addition of the amine-terminated linker will add +143 to the mass of the DNA.

Addition of the disulfide linker will add +340 to the mass of the DNA.

Cleavage of the disulfide (this will occur after DTT treatment and sometimes during the MS ionization process) will result in a mass that is +231 more than the mass of the DNA.

Several peaks corresponding to addition of sodium (+23) are sometimes observed, especially when taking the mass spectrum of the DNA + disulfide.