Sephadex spin-column protocol for cleaning PCR products

To make new sephadex: add 5 g of sephadex G-50 powder to 75 ml of ddH_2O. Swirl to mix fully. Allow new sephadex solution to hydrate for at least 45 min before using.

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1. Place empty spin columns in old 1.5 ml collecting tubes.

2. Swirl sephadex solution to suspend. Fill each column with 800 µl of sephadex sol.

3. Allow water to drain (~5 min) from columns until sephadex becomes visible.

4. While columns are draining, label 1.5 ml eppendorf tubes.

5. Centrifuge collecting tubes with columns inside at 3,800 rpm for 2 min. Plastic nubs should face up/out.

6. Remove column carefully and place them in labeled eppendorf tubes.

7. Transfer each PCR sample to the appropriate column. Be very careful when performing this transfer. In transferring the solution, try to place the solution in the center of the column matrix without touching it.

8. Place collection tubes with colums inside in the centrifuge so that the hinged lid of the tube faces the center of the rotor and the plastic nub of the column faces out. Centrifuge at 3.8 rpm for 2 min.

9. Columns and collection tubes can be reused after cleaning by spraying the inside vigorously with distilled water or boiling them for a few min using a microwave.

PCR cleanup with ExoSAP

The Exo-SAP treatment degrades leftover PCR primers and unincorporated sNTPs.

1. For each reaction mix:		x ____
Sterile water	7.5 µl	_____
Exonuclease I (10 U/µl)	0.5 µl	_____
Shrimp Alkaline Phosphatase (1 U/µl)	0.5 µl	_____

2. Aliquot 8.5 µl of this mixture into tubes or plate wells.
3. Add 8 µl of your PCR product.
4. Run ExoSAP profile in the PCR machine:
   37° C for 30 min (leftover primers are degraded)
   80° C for 15 min (enzyme is degraded)
5. Use 1-3 µl for sequencing reaction.

CYCLE SEQUENCING

Cycle sequencing reaction:

dH_2O (to adjust total volume of 20 µL)	___ µL
DNA template (25-100 fmol see below*)	___ µL
5x Sequencing buffer	4.0 µL
Primer (5µM)	0.6 µL
Betaine (5M, optional)	4.0 µL
DTCS Quick Start Master Mix	2.0 µL
	20.0 µL

* The amount of template needed will depend on the size of the PCR fragment.

Table to calculate amount of DNA to add to the sequencing reaction

Size (kilobase pairs)	ng for 25 fmol	ng for 50 fmol	ng for 100 fmol
0.2	3.3	6.5	13
0.3	4.9	9.8	20
0.4	6.5	13	26
0.5	8.1	16	33
1.0	16	33	65
2.0	33	65	130
3.0	50	100	195
4.0	65	130	260
5.0	80	165	325
6.0	100	195	390
8.0	130	260	520
10.0	165	325	650
12.0	195	390	780
14.0	230	455	910
16.0	260	520	1040
18.0	295	585	1170
20.0	325	650	1300
48.5	790	1500*	1500*
For ssDNA, the values (ng) should be divided by 2. *Use no more than 1.5 µg of template DNA.			

Thermal cycling conditions:

96° C	20 sec.	
50° C	20 sec.	
60° C	4 min	x 30 cycles
4° C	hold	

* To increase signal one may increase cycles to 45.

ETHANOL PRECIPITATION

1. Prepare a stop solution cocktail for each sample to stop the sequencing reaction. This solution needs to be prepared fresh. x ____

3M NaOAc pH 5.2	2.0 µL	_____
100 mM Na_2EDTA pH 8.0	2.0 µL	_____
20 mg/mL glycogen	1.0 µL	_____

2. Add 5 µL of the prepared stock to each well.

3. Add **60 µL of ice cold 95% EtOH** to each well, cover plate with a foil seal-it self-adhesive cover, vortex samples and invert covered sample plate 3-5 times.

4. Centrifuge sample plate in a Beckman Allegra 25R bench centrifuge at 6,130 xg **(5,700 rpm) for 10 minutes** to precipitate DNA. If this centrifuge is not available, samples must be centrifuged at a minimum of 3,000 xg for 30 minutes in a refrigerated centrifuge with a rotor that can accommodate 96 well plates.

$g = (1.118 \times 10^{-5}) R S^2$

g = relative centrifugal force
R = radius of the rotor in cm
S = speed of centrifuge in revolutions per minute

5. Remove plate and invert to decant excess EtOH. Gently blot the plate on an absorbent pad such as folded paper towels. Invert only once to decant EtOH and then turn plate right side up to proceed with the following wash step. Be cautious not to disrupt the pellet.

6. Add **200 µL of ice cold 70% EtOH** to each well. Centrifuge at speed described above for **5 minutes**; decant the excess EtOH as described in step 5. After this there is no need to cover the sample plate with the seal-it foil. **Repeat** this step.

7. After the second wash keep the plate inverted and gently place the inverted plate onto the plate holder in the bucket rotor plate carrier with 3-4 folds of paper towels below the wells to absorb the excess EtOH. Centrifuge the inverted sample plate for **30 seconds** at **200 rpm**.

9. Vacuum dry the samples for 10-20 minutes. Optionally, allow the samples to air dry on the bench for 30-40 minutes. Visually inspect the pellets to be sure they are sufficiently dried.

10. Resuspend each sample in **30 µL of deionized formamide** (also known as SLS).

11. Overlay each of the resuspended samples with one or two drops of mineral oil and load the sample plate into the CEQ 2000XL.