Preparing samples for electrophoretic separation

Electrophoretic separation

For this service, clients submit the extension products after cycle sequencing (fluorescent labelling) and subsequent post sequencing clean-up have been performed. Listed below are the recommended protocols for sequencing reaction set up, and post reaction clean up.

Sample names (< 10 characters long)

Please label all sample tubes with the sample name and number on the lid of the 1.5ml microfuge tube and your full name on the side of the tube.
The following characters are not recognized by the sequencing software: \ / | : * ? < > "space" so please avoid their use.

Quality of template

Template purity is a key contributor to the quality of the resultant sequence data. It is preferred that all DNA templates are post-extraction purified using a column-based DNA purification kit that ensures that residual RNA, salt, protein and other contaminating chemicals are removed. *ExoSAP-IT* for PCR purification and *TempliPHI* for plasmid amplification routinely produce very high quality DNA for sequencing as does the *Alkaline Lysis PEG precipitation method*

Quantity of template

Using the correct amount of template is critical for achieving high quality results. Too little or too much DNA will reduce the length of read and the quality of base calls. Refer to table 1 for the recommended amount of template required. The volume of template used should not exceed 6ul as we have found that less concentrated templates produce lower quality sequence reads. It is recommended to quantitate templates by gel electrophoresis with a known mass standard or by nanodrop.

Table 1. Recommended amounts of template and primer for sequencing reaction

Template	**Quantity**
Primer	3.2 pmol
Double-standed template	200-400ng
Single-standed template	50-100ng
BAC	500-1000ng
Bacterial genomic DNA	2-3 μg
PCR product:	
100-200bp	5-10ng
200-500bp	10-25ng
500-1000bp	25-50ng
1000-2000bp	50-100ng

Sequencing difficult templates

The addition of 5% DMSO or 1M Betaine during sequencing cycling is recommended when sequencing templates containing secondary structure or high GC ratios.

Table 2. Sequencing primers recommendations

Primer length	Primers should be at least 18 bases long to ensure good hybridisation
Single nucleotide runs	Avoid runs of an identical nucleotide, especially runs of four or more Gs
GC content	Keep the G-C content in the range 30–80% For primers with a G-C content less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the melting temperatures (Tm) above 50°C
Melting temperatures	For cycle sequencing, primers with Tm > 50°C produce better results than primers with lower Tm
Secondary hybridisation site	Use of primers longer than 18 bases also minimises the chance of having a secondary hybridisation site on the target DNA.
Secondary structure	Avoid primers that have secondary structure
Primer dimers	Avoid primers that can hybridise to form dimers
Concentration	Use relatively fresh stocks of primers diluted to 3.2pmol/ul in MilliQ water
Degenerate primer	Avoid degenerate primers

Table 3. Primers available from facility

Upon request the following primers are available from the facility at 3.2 pmol/ul (um) free of charge.

Primer	**Sequence**
-21 M13 Primer	5' TGT AAA ACG ACG GCC AGT 3'
Reverse Primer	5' TCA CAC AGG AAA CAG CTA TGA C 3'
T3 Primer	5' ATT AAC CCT CAC TAA AGG GA 3'
T7 Primer	5' TAA TAC GAC TCA CTA TAG GG 3'
SP6 Primer	5' TAT TTA GGT GAC ACT ATA G 3'

Table 4. Preparing sequencing reactions

For each reaction add the following reagents to a separate tube:
(Note: final dilution of BigDye is 1:8)

Reagent	**Quantity**
BigDye V3.1	1μl
5X BDT buffer	3.5μl
Template (refer "Template Quantity" above) Primer 3.2pmol MilliQ water	15.5μl total
Total reaction vol.	20μl

Table 5. Cycle Sequencing conditions

1	Place tubes in thermal cycle and set volume to 20μl
2	Initial denaturation Rapid thermal ramp to 96 °C 96°C for 1 min
3	Repeat the following for 25 cycles
	Rapid thermal ramp* to 96 °C 96 °C for 10 sec Rapid thermal ramp to 50 °C 50 °C for 5 sec Rapid thermal ramp to 60 °C 60 °C for 4 min
	*Rapid thermal ramp is 1°C/second
	Rapid thermal ramp to 4°C and hold until ready to purify
	Proceed to, "Purifying Extension Products"

Table 6. Purifying Extension Products by ethanol/sodium acetate precipitation

1	Remove sequencing reactions from thermal cycler
2	Combine the following for each reaction 2μl sodium acetate pH4.6 50μl of 95% ethanol
3	Add the 20μl reaction volume to the ethanol/sodium acetate mix
4	Vortex. Precipitate for 15-30 minutes at room temperature
5	Centrifuge at 13,000 rpm for 20-30 minutes (tube hinge outward)
6	Carefully aspirate the supernatant using a 200μl pipette tip until all ethanol is removed
7	Add 250ul of 70% ethanol
8	Vortex
9	Centrifuge at 13,000 rpm for 10-15 minutes (tube hinge outward)
10	Carefully aspirate the supernatant using a 200μl pipette tip until all ethanol is removed
11	Dry tubes in a heat block at 90°C for 2-5 minutes
12	Close lids and store, protected from light, at 4°C until ready for electrophoresis

Submitting samples in 96-well tray

Please use Axygen PCR trays when submitting samples for electrophoresis in 96-well plate format. Samples should be loaded in consecutive order starting from A1, B1. C1 to H1 then A2, B2, C2 to H2 etc.