DNA SEQUENCING PROTOCOL (DS group)

Jill Toombs, 2003

SAFETY NOTES

Wear fastened up lab-coat, disposable latex/nitrile gloves, and safety goggles. Read the appropriate MSDS sheets before beginning the method. Work in a well ventilated area and use laboratory fume extraction cabinet where recommended. Know how to dispose of hazardous waste before starting the method.

Hi-Di formamide - may cause harm to the unborn child
DMSO - Has been found to cause cancer in lab animals. Also may cause adverse mutagenic or teratogenic effects.
ExoSAP-IT - Irritant

PCR amplification (10µl reaction volume)

- Correct primer design is essential: Preferably use ‘Primer 3’, ‘Primer detective’, or ‘VNTI’ software.

- Annealing temperature Tm – 5 (try to achieve an annealing temp of ~65°C)

- 9µl PCR master mix (Use A/Gold if possible or Qiagen Taq polymerase) + 1µl DNA (25ng)

Method

Per 1 reaction [Note: Use 0.5µl (5%) DMSO if high GC content]

<table>
<thead>
<tr>
<th>7.05µl</th>
<th>H2O</th>
<th>Final conc.</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0µl</td>
<td>10 X buffer</td>
<td>1x</td>
<td></td>
</tr>
<tr>
<td>0.6µl</td>
<td>MgCl2 (25mM)</td>
<td>1.5mM</td>
<td></td>
</tr>
<tr>
<td>0.1µl</td>
<td>dNTPs (20mM)</td>
<td>0.2mM</td>
<td>Abgene AB-0196</td>
</tr>
<tr>
<td>0.1µl</td>
<td>forward primer (10µM)</td>
<td>0.1µM</td>
<td></td>
</tr>
<tr>
<td>0.1µl</td>
<td>reverse primer (10µM)</td>
<td>0.1µM</td>
<td></td>
</tr>
<tr>
<td>0.05µl</td>
<td>Taq (5U/µl)</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

9µl

Add 1µl of genomic DNA @ 25 ng/µl and proceed to PCR

Run 2µl of PCR product on a 1.5% or 2% agarose gel to monitor reaction. The reaction must be specific with no visible evidence of primer dimer.
**NOTE:** The following must be carried out in a POST-PCR area using POST-PCR pipettes etc.

**EXOSAP-IT PCR Clean-up**

*NB: EXOSAP-IT must be kept on ice at all times when removed from the freezer and returned immediately to the freezer after use.*

**Method:** (Plate method): Per 1 reaction

Aliquot 2µl of EXOSAP-IT into each well in a 96 well plate (ABgene AB-0731) (ie. one well for each reaction)

Add 5µl of neat PCR product to the appropriate well containing EXOSAP-IT. Mix gently pipetting up and down in the pipette tip three times. Seal the plate with adhesive PCR film (ABgene AB-0558)

Place plate into a 9700 PCR machine and run as follows:

37°C for 15 minutes (enzyme incubation)

80°C for 15 minutes (enzyme inactivation)

4°C soak for a mimimum of 5 minutes

Once the plate has cooled at 4°C, remove the plate asap from the PCR machine. Spin the plate briefly in the Centaur 2 post-PCR centrifuge and proceed with cycle sequencing.

**CYCLE SEQUENCING**


- Big Dye Kit must be thawed at room temperature (*do not heat*!), kept on ice during use, protected from light and returned to the freezer immediately after use.

**Method**

Dilute purified PCR product 1:4 in MilliQ grade water in a separate 96 well AB-0731 plate: (2µl of PCR + 6µl H₂O). NOTE: The dilution factor may vary depending on the strength of the PCR product - refer to Figure 1 for examples.
**Per 1 cycle sequencing reaction (5µl)**

[Note: may need to use 5% of DMSO (0.25µl) if high GC content]

2.0µl Big Dye Mix  
0.4µl primer (2µM)  
0.6µl H₂O  
___  
3.0 µl

Aliquot mix in 3.0 µl amounts into a MicroAmp Optical 96-Well reaction plate (Applied Biosystems N801-0560) and add 2.0 µl of diluted PCR product from above. Seal the plate with adhesive PCR seal (ABgene AB-0558) and spin at 1500rpm for 30 seconds in a Centaur 2 Post PCR Centrifuge. Place into a 9700 PCR machine and run as follows:

**Cycling conditions**

96°C/20s followed by  
25 cycles of  
96°C/10s  
50°C/5s  
60°C/4min followed by  
4°C Soak until required

Remove plate from PCR machine and spin briefly. Remove the adhesive PCR film.

**Add 14µl of H₂O to each 5 µl cycle sequencing reaction and mix by pipetting up and down three times.**

**ETHANOL PRECIPITATION (Plate method)**

**Note:** Ethanol should be prepared fresh as follows:  
For 95% ethanol, add 2.5mls milliQ water to 47.5mls AR quality ethanol  
For 70% ethanol, add 15mls milliQ water to 35mls AR quality ethanol

Prepare the following mix (scale-up depending on number of samples)  
**Per 1 sample (prepare freshly)**

49µl 95% ethanol  
3 µl 2M sodium acetate (Applied Biosystems)  
___  
52µl
Using a repeater pipette, add 52µl of ethanol / sodium acetate mix to each 19µl sample from above. Cover plate with an adhesive seal (ABgene AB 0-580).

Manually invert the plate 10 times to mix.

Leave the plate at room temperature for 15 minutes to precipitate extension products.

Spin plate at 4000 rpm for 45 min in a Centaur 2 (post-PCR) centrifuge. During the last 5 minutes of spin time, remove Hi-Di formamide from the freezer to thaw at room temperature.

Remove adhesive seal and invert the plate onto a paper towel.

Transfer inverted plate onto fresh paper towel and spin at 1500 rpm for 30 seconds in a Centaur 2 (post-PCR) centrifuge.

Using a repeater pipette, add 150µl of 70 % ethanol to each well and cover with an adhesive seal (ABgene AB-0580).

Manually invert the plate 10 times to mix.

Spin plate at 4000 rpm for 10 min in a Centaur 2 (post-PCR) centrifuge.

Remove adhesive seal and invert the plate onto paper towels.

Transfer inverted plate onto fresh paper towels and spin at 1500 rpm for 30 seconds.

*NOTE*: At this stage, the plate may be frozen in the -20 freezer (post-PCR) until required. However, it is preferable to proceed immediately to the next step.

Add 12µl of Hi-Di formamide to each well cover with an adhesive seal (ABgene AB-0580). Vortex the plate for 10 sec (use AEH group vortex which has an adaptor for vortexing a plate).

Place the plate into a 9700 PCR machine and, without shutting the lid, denature for 5 minutes at 95°C.

Remove the plate from the PCR machine and spin for 30 seconds at 1500rpm.

Remove the adhesive seal and reseal the plate using a grey rubber sequencing lid (stored in the cupboard to the bottom right of the 3100. If there are none in the cupboard then remove one from a previously run plate, wash thoroughly with tap water and blot dry with tissue paper).

Place the plate on ice prior to loading on 3100 sequencer.

*Following sequencing*, the plate must be removed from the 3100 sequencer and disposed of into the appropriate yellow sharps bin in the left fume extraction cabinet in the QUB research lab (retain and wash the grey rubber seal).