**BigDye Sequencing Reactions**

*(10/18/06, SWH)*

<table>
<thead>
<tr>
<th>Rxn Size</th>
<th>Primer (3.2 uM)</th>
<th>Buffer (2.5X)</th>
<th>BigDye mix</th>
<th>DNA + H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/16</td>
<td>1 ul</td>
<td>3.50</td>
<td>0.500</td>
<td>5</td>
</tr>
<tr>
<td>1/20</td>
<td>1 ul</td>
<td>3.60</td>
<td>0.400</td>
<td>5</td>
</tr>
<tr>
<td>1/24</td>
<td>1 ul</td>
<td>3.67</td>
<td>0.333</td>
<td>5</td>
</tr>
<tr>
<td>1/28</td>
<td>1 ul</td>
<td>3.71</td>
<td>0.286</td>
<td>5</td>
</tr>
<tr>
<td>1/32</td>
<td>1 ul</td>
<td>3.75</td>
<td>0.250</td>
<td>5</td>
</tr>
</tbody>
</table>

**Cycling Profile**

- 95°C initial denaturation (2'); 25X [95°C (10 s); 50°C (5 s); 60°C (4')]; 20°C hold (4°C if can't remove plate on completion).

**Clean-up: Spin plate. Use EtOH²⁵ or DyeEx plate (bring samples to ~20 ul; prepare DyeEx plate¹⁷; load samples).**

**Final Prep:** Dry samples @ 70°C, in a PCR block or a SpeedVac. Resuspend all samples in 15 ul of formamide. For "used" DyeEx plates, resuspend overnight @ 4°C.

**Notes:**

1. PCR template: data unreadable (strand-slipage) after pure poly-"single-base" region (~10 bases)⁵⁵; must sequence both ways.
2. Purified M13 PCR product (from bacterial colony pick) gives adequate sequence data, but readable data stops post poly-A tail.
3. Use of PCR primer loses only ~10-50 bp of initial sequence and extends ~100 bp farther than when M13 primers are used.
4. The M13 primers capture ~70 bp of vector sequence; thus, add ~100 bp to product size to select BigDye reaction size. Further, primer amplifying across insert toward poly-A tail gives cleaner, longer sequence (but, orientation of insert is usually unknown).
5. **(A)** A-rich mid-region of Alu isn't a problem; **(B)** Use "reverse" PCR primer only if sequence following poly-A tail is required.
6. PCR product must be a single band (either before or after gel purification); otherwise, cloning is required.
7. **(A)** Residue from filter in Wizard kit gives false spec. reading (~30 ng/ul); thus, PCR product must be gel-quantified (estimated). **(B)** Ideally, purify enough PCR product for spec. reading, & make serial dilution reference photo down to 1 ng in clean 2% gel.
8. Elute DNA w/50-100 ul. If loading in gel, remove residual EtOH @ 50°C; otherwise, vapor can damage unit.

**Determining required length of good quality, post-primer sequence:** Use least costly associated size of BigDye reaction²¹,²²,²³

- 500-650 bp: 1/32X
- 500-700 bp: 1/28X
- 650-725 bp: 1/24X
- 650-750 bp: 1/20X
- 700-800 bp: 1/16X

**Alternatives:**

- If band is barely visible from 1 ul & clearly visible from 3 ul, 3 ul will give good sequencing results.
- Mid- to low-range DNA mass values give best overall results (read length & signal strength); ~400-600 ng (vector) or ~14-20 ng (PCR) can shorten read length to minimum stated values, especially for reactions @ ≤1/24X. However, to minimize quantitation efforts, consider developing a standard dilution, based on typical DNA mass produced from mini-preps or each PCR primer pair.²⁶
- For more DNA volume, use different associated size of BigDye reaction.

**References:**

1. Mostly adopted from sequencing Alus: Do you need Sequence on BOTH sides of a poly-"single-base" region (e.g., poly-A tail or neighboring repetitive element)?
2. Use "forward" PCR primer with 50-250 ng DNA.²⁴
3. Use either (or both) M13 Vector primer⁴ with 50-250 ng DNA.⁹
4. Use "forward" PCR primer with 50-250 ng DNA.²⁴
5. Use "reverse" PCR primer only if sequence following poly-A tail is required.
6. The M13 primers capture ~70 bp of vector sequence; thus, add ~100 bp to product size to select BigDye reaction size. Further, primer amplifying across insert toward poly-A tail gives cleaner, longer sequence (but, orientation of insert is usually unknown).
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