

I. ABI BIGDYE CYCLE SEQUENCING REACTION

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| <p>REAGENTS <u>Reagents should be kept on ice</u></p> <p>BigDye Ready Reaction Mix (BD)</p> <p>Big Dye Dilution Buffer (2.5X BDDB)</p> <p>Primer (10uM)</p> <p>DNA (gel-purified PCR product; clean plasmid prep) Suggested DNA concentration per sequencing rxn:</p> <p>-15-20ng per 100bp of <i>Clean</i> PCR product</p> <p>-200 – 400 ng plasmid</p> <p><u>BD is light sensitive so keep it in the dar</u></p> | <p>Recipes:</p> <p>2.5x BDDB:</p> <p>-200ul 1M Tris HCl pH9</p> <p>- 5ul 1M MgCl₂</p> <p>-milli-Q H₂O to 1ml</p> |
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For 5uL reactions using 1:1.5 ratio of BD:BDDB (some people use different ratios)

- Calculate the number of wells you have for sequencing by multiplying the number of samples times the number of primers.
I.e.: If you have 4 samples and 2 primers: $4 \times 2 = 8$ wells.
- Number each well (Best to use a multiwell plate) from 1-8 (for this example) and write up a sample sheet with the order (1.sample1primer1, 2.sample1 primer 2,...).
- Prepare a Master Mix for each sequencing primer using the following measurements per sample; use a slop factor. **FOLLOW THE EXAMPLE IN THE TABLE BELOW**

0.8uL of BD
1.2uL of 2.5X BDDB
0.2uL of 10uM primer
0.2uL of 25mM Mg⁺⁺

Example: If you have 4 samples (+ 1 slop factor=5 samples) and two (2) primers (A and B) you will have:

| | Master Mix 1 (<u>Tube 1</u>) | Master Mix 2 (<u>Tube 2</u>) |
|-----------------------|---|---|
| BD | $5 \times 0.8 = 4.0\text{uL of BD}$ | $5 \times 0.8 = 4.0\text{uL of BD}$ |
| 2.5X BDDB | $5 \times 1.2 = 6.0\text{uL of BDDB}$ | $5 \times 1.2 = 6.0\text{uL of BDDB}$ |
| 25mM Mg ⁺⁺ | $5 \times 0.2 = 1.0\text{uL of Mg}^{++}$ | $5 \times 0.2 = 1.0\text{uL of Mg}^{++}$ |
| 10uM primer | $5 \times 0.2 = 1.0\text{uL of primer A}$ | $5 \times 0.2 = 1.0\text{uL of primer B}$ |

4. Add 1.5uL template + 1.1uL Milli-Q H₂O to each numbered well.

If template concentration is low, ie. <30ng of whole region, use 1.7uL template + 0.9uL Milli-Q H₂O

5. Add 2.4uL of Master Mix into each numbered well.

6. Put on thermal cycler:

THERMAL CYCLER (BIGDYESM program on MJ)

| Stage | Temperature | Time |
|--------------|-------------|---------|
| Denaturation | 95°C | 10sec. |
| Annealing | 50°C | 5sec. |
| Extension | 60°C | 4 mins. |

REPEAT CYCLES 35X, FOLLOW BY HOLD AT 4 °C

This program takes 3.5 hrs.

II. PRECIPITATION

1. Add 20uL of 75% Isopropanol. Vortex briefly by hand.
2. Let sit at room temperature for 15 mins.
3. Centrifuge at 4000rpm for 30mins.
4. **Immediately** when spin has stopped (if not possible quick-spin before removing from centrifuge): Remove plate seal. Quick-spin plate upside down on a tissue layer(kimWipes) for 6 seconds; repeat twice changing tissue layers each time to ensure complete removal of supernatant.
5. Add 75uL EtOH 70% (ice cold ethanol)
6. Centrifuge at 4000rpm for 5 mins.
7. **Immediately** when spin has stopped (if not possible quick-spin before removing from centrifuge): Remove plate seal. Quick-spin plate upside down on a tissue layer(kimWipes) for 6 seconds; repeat twice changing tissue layers each time to ensure complete removal of supernatant.
8. Leave to dry at room temp for 20 minutes on top of your bench. Or use: FOREVER2 program (on PCR machine):
55C for 15 minutes
22C for 3 minutes
9. If not sequencing immediately, store at -20°C.

III. Denature (after precipitation)

1. Re-suspend samples in 2.5uL of de-I Formamide/loading buffer mix.
2. Denature using PCR machine in seq. room:
program DENATFR:
95°C for 5 minutes
3. Take out and put **IMMEDIATELY** on ice.
4. Leave on ice until ready to load into membrane comb.

Recipe: **Formamide Loading Buffer:**

0.05g Blue Dextran in 1mL of 25mM EDTA
Add to De-I Formamide in ratio 5:1
i.e. 200uL EDTA + 1000 Formamide

IV. Loading comb:

SUGGESTION:

In order not to waste tips (0.5-10uL) have the running buffer (1200mL 1X TBE) ready by the time you load the membrane comb so you may be able to rinse the tip every time you add in your samples. If loading comb ahead of running gel time, then it's not necessary.

Running buffer recipe:

120mL 10X TBE + 1080mL MQH₂O
OR 240mL 5X TBE + 960mL MQH₂O

1. Add 0.9uL of denatured samples into each well of sample tray.
2. Once all samples are loaded in tray, put comb into sample tray (each tine should fit into each well). Let soak for 10 seconds.
3. Comb is ready to be loaded in the gel. If not using immediately, place comb into its plastic cover and put in freezer until ready to run gel.

****Membrane comb usage:**

1. Have no more than one comb in circulation.
2. Label your comb with your name or project.
3. Mark your comb with a check mark after every use.
4. After five times of usage dispose and use a new one.

V. ABI SEQUENCING GEL (Gel Pouring)

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| <p>THERMOPAGE GEL 14.4g Urea (aliquoted in falcon tube) 4ml 10x TBE 5ml Thermopage (40%) 19.8ml Milli-Q H₂O 200ul 10%APS (at -20°C) 20ul TEMED (at +4 °C)</p> | <p>10x TBE: 104g Tris base 56g Boric Acid 8g EDTA Na take up to 1L w/ Milli-QH₂O (DO NOT USE IF PPT. VISIBLE, if so, filter again) 10% APS: 0.03g APS (aliquoted in 200ul tube) 270uL Milli-Q H₂O</p> |
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ALL GLASSWARE MUST BE RINSED WITH MQ-H₂O BEFORE USE.

PREPARING + POURING GEL:

1. Add TBE, Thermopage & water to Urea in falcon tube. Mix gently to dissolve.
2. Filter gel through 0.45 micron filter.
3. De-gas solution under vacuum (tighten filter assembly after solution has passed through) Let sit **in vacuum** for 15 minutes (time to prepare your plates) **Make sure de-gaser has two orange round rubber gaskets attached correctly.**

ASSEMBLING PLATES:

1. Wash plates carefully with LOTS of hot water. Ensure no acrylamide residue. Rinse with distilled water.
2. Rinse with MQ-H₂O. Leave to air-dry in rack (20 mins or as long as it takes).
3. Assemble plates with gel caster:
 - a. Adjust gel caster to fit your plates' width with the side knob. (**it is already set**)
 - b. Place bottom plate in lower rail of gel caster with the letters facing down (inside part upward)
 - c. Add droplets of water to spacers (only to the side that touches the lower plate) and align each spacer to the edge of the glass.
 - d. Place top plate face down (letters facing up), in the higher rail of gel caster.
 - e. Slide the top plate towards the bottom plate till they overlap by about an inch.

***Plates are ready for gel pouring.**

4. Release vacuum (turn off vacuum and take off hose SLOWLY)
5. Transfer gel solution from filter to 50/100 beaker.
6. Place gel solution on ice if you're not going to pour gel immediately
7. When plates are ready for pouring:
 - a. Add quickly **IN THIS ORDER**: 200ul of 10%APS and 20ul TEMED. Swirl beaker while adding, to mix well.
8. Pouring gel:
 - a. Slowly pour gel mix close to the center of the leading edge of the top plate. Keep sliding the top plate forward until the whole plate area is full of gel.
 - b. If there are any bubbles at any time while pouring the gel, slide top plate back until bubbles disappear.
9. After **5mins**. Apply damp kimwipe & clingfilm.
10. Leave to polymerize **2 hrs**. Ensure remaining solution sets in beaker after 20 mins.

VI. LOADING + RUNNING GEL:

1. Make up 1200mL 1X TBE (this is the running buffer)
 - i. 120mL 10X TBE + 1080mL MQ-H₂O **OR** 240mL 5X TBE +960mL H₂O
2. Unwrap plates and clean upper and lower parts of plates (laser read-region and upper-buffer-tank region) with Milli-Q water. Ensure no acrylamide residues on each part, if so use shark's tooth comb to pull them out.
3. Drain well area with kimwipe by tilting plates to the side (let kimwipe soak the water off well region).
4. Place plates in grey gasket. FASTEN KNOBS.
5. On ABI sequencer, place lower buffer chamber into machine and plug in wire.
6. Place gasket into machine and FASTEN KNOBS.
7. Close ABI door.
8. On computer:
 - a. double click on ABI prism 377
 - b. go to FILE, NEWclick on Sample sheet (type in your sample's orders)
 - c. Save and close.

 - d. MAKE SURE THIS STAYS THE SAME FOR A SEQUENCING GEL:

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| Plate check E | Pre-run module – ‘Seq PR 36E-2400’ |
| Run module - ‘Seq Run 36E-24’ | Collect time – 3.5 hrs |
| Sample Sheet – “whatever” | Well-to-read distance – 36 cm |
| Lanes - 48 | Instrument File-‘dRho_BD 07/2001’ |

- e. You should only change your sample sheet and the hours to be run.
 - f. click on Seq. run (or GeneScan if doing microsats)
 - g. Start PLATE CHECK
9. While on PLATE CHECK, find your comb (or load it if you still haven't). In scan region, four colored lines should appear. They should be straight. If there are high peaks, hit PAUSE, wait till it says OK (click OK), open door and check that the read region is CLEAN (take off plates if necessary). Close door again. Choose RESUME and wait till lines appear.
 10. When done, choose CANCEL, then click on TERMINATE.
 11. Open ABI door (when it says it's OK), add 1X TBE buffer into lower buffer chamber through the side.
 12. Load 400uL Ficoll with a pipette into the well region on upper part of plates.
 13. Load membrane comb into well region; use a 20-degree angle while loading comb.
 14. Attach upper buffer chamber, make sure green rubber band is in place, plug in wire.
SECURE WITH KNOBS.
 15. Pour 1X TBE to upper buffer chamber.
 16. Unfasten PLATE knobs and place HotPlate near upper buffer tray so the lower part won't have any pressure. Fasten knobs, connect hoses and plug in the ground wire (HotPlate wire).
 17. Close ABI door and on computer:
 - a. click on RUN
 - b. Let run for one (1) minute.
 - c. Have a bucket ready with MQ-H₂O to rinse the comb later.
 - d. On computer click PAUSE, wait till it says OK.
 - e. Open ABI door; take comb out. Put it in bucket with water and let it rinse for 5 minutes.
 - f. Place upper buffer chamber's lid on. Close ABI door. And hit RESUME on computer. Gel is now running.

- g. After five minutes, take comb out of water bucket and pat dry with kimwipe.
Place a layer of kimwipe between its plastic holder and put in comb.
- h. Place comb between heavy books or on any flat surface with a flat holder on top to the comb wont bend.

18. **Clean up.**

VII. POST RUN (cleaning apparatus)

1. Remove hot plate.
2. Fasten clips again so that plates are secure.
3. Remove gel apparatus (grey tray with upper buffer chamber) from machine; take it to the sink, and empty top tank.
4. Take plates from grey frame. Let sit horizontally on foam to remove spacers.
5. Separate plates, slowly & carefully using razor blade along edges of plates.
6. Remove gel using kimwipe.
7. Wash plates well with PLENTY of hot water. Try **NOT** to use soap. Use fingers only to avoid scratching plates if there are still any acrylamide residues left.
8. Rinse plates with MQ-H₂O, ensuring no acrylamide residues.
9. Rinse spacers tap water then MQ-H₂O. **Do not use soap.**
10. Leave all to air dry.
11. Make sure all other apparatus are washed (with water only) and rinsed with MQ-H₂O.