Plate Protocol: Precipitation of 10-μl PCR Reactions  (30 October 2013)

1) Remove 96-well plate [Note #1] from thermocycler; spin (3800 rpm or 2500 RCF; ≤1 min).

2) Add 5 μl EDTA (70 mM, pH 8) to each well (for a 20 μl reaction, use 5 μl @ 125 mM & increase EtOH volumes, including wash, to 60 μl); ensure that EDTA droplet enters sample before Step 3.

3) Add 30 μl 100% EtOH to each well [Note #2].

4) Seal plate [Note #3]; mix by repeatedly (4X) inverting and vigorously shaking plate.

5) Incubate @ room temperature (RT; 15 min).

6) Spin plate (Program 1: 3800 rpm or 2500 RCF, 20°C, 15-30 min). Proceed to Step 7 immediately; otherwise, re-spin plate for additional 2 min prior to Step 7. [Note #4]

7) Cover plate with “foil cap” (with thick pad of Kimwipes inside cap) and put upside-down in centrifuge plate bucket. Spin plate (Program 2: 150 RCF, 1 full minute). [Notes #5-7]

8) Add 30 μl 70% EtOH to each well (for 20 μl reactions, increase to 60 μl; see Step 2); reseal plate.

9) Spin plate (Program 3: 3800 rpm or 2500 RCF, 20°C, 5-10 min).

10) Repeat Step 7.

11) Dry plate to remove traces of EtOH (in thermocycler @ 70°C, ~5-10 min; or in SpeedVac).

12) Resuspend DNA.

- **BigDye Sequencing reactions**: add 15 ul ABI® Hi-Di formamide to each well; seal plate; briefly vortex; spin plate @ 2,500 rpm (≤1 min). Refrigerate plate until samples are placed on 3130XL sequencer. [Note #8]

- **Un-sequenced PCR products**: Add sufficient TVLE such that 1-3 μl of template will be appropriate for a BigDye sequencing reaction; see How much DNA to use in a sequencing reaction?. [Note #9]

**Note 1**: You can also process 0.2-ml tubes if you put them in a 96-place tube rack, with blank tubes on the other end of the rack for balance. For 1.5-ml tubes, see alternative protocol.

**Note 2**: Store EtOH solutions in a low-humidity environment (e.g., fridge or freezer) to prevent absorption of water from the air; your DNA won’t precipitate if the % of EtOH drops too low.

**Note 3**: If the ‘droplets’ do not ‘fly about’ in wells, you’re being too timid and not truly mixing contents; a PCR mat or caps are the most reliable seals, but some types of adhesive seals can fully seal each well.

**Note 4**: Signal strengths from 15 vs. 30 min spins are ~ identical; however, a 15-min spin loses ~10-20 bp of sequence close to the primer (vs. a 30-min spin). Signal strength drops with shorter spin times (e.g., 10 min). ABI recommends 4°C spins; RT actually works better.

**Note 5**: The “Foil Cap” is a sheet of aluminum foil shaped to fit like a cap over the 96-well plate; it lets you flip the 96-well plate upside-down without spilling EtOH and potentially cross-contaminating samples. Alternatively, you can turn the centrifuge plate bucket (with thick Kimwipe insert) upside-down, insert plate face-up, flip assembly over, and spin... but, more EtOH will end up in the centrifuge.

**Note 6**: Steps 7 & 10 have been successful using high rcf by accident; but, it’s not a good idea or necessary.

**Note 7**: Residual EtOH contains numerous unincorporated dye terminators (UDT’s). Drying the EtOH does not remove UDT’s; if not removed, UDT’s degrade sequence data (see Why do massive peaks occur ~50-70 bp into my data?). Inadequate spin time may leave residual EtOH in the wells; however, some users reported problems with product recovery when spinning flexible plates upside-down. If this occurs, consider using: (A) ~5 seconds @ ~200-300 rcf, or (B) the full minute @ ~150 rcf with the flexible plate in a rigid 96-place rack — but, ensure that all EtOH has spun out before proceeding.

**Note 8**: Other formamide brands may have water, contaminants, or improper buffering, leading to poor signal. Vortexing is not essential, but may be helpful if the plate will be loaded immediately on the sequencer.

**Note 9**: DNA recovery should be >80-90%; thus, estimate resuspension volume based on brightness of raw PCR products in a gel, keeping in mind that some of the original reaction has already been consumed. For example: Assume ran 5 ul of a 25-ul PCR reaction in the initial agarose gel, with band brightness suggesting that 1-ul of the raw product would be sufficient for a BigDye reaction: 80% of original product remains, so resuspending in ~17-ul (i.e., ~85% * 20 ul) should retain same concentration; if we want to add 2-ul to each BigDye reaction, resuspend in ~34-ul.