Protocol for Preparing DNA for Ready to Load [RTL] Sanger Sequencing

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Summary

This is a procedure for amplifying, manipulating, and cleaning up DNA in preparation for Sanger Sequencing at Cornell's Life Sciences Core Laboratories Center (CLC), using their Applied Biosystems 3730 Sequence Analyzer. More information about the CLC, their services and requirements can be found on their website: http://www.brc.cornell.edu/brcinfo/?p=rtl, and by calling (607) 254-4857.

Materials

PCR reagents
Agarose (low to medium grade)
Exonuclease I (Exo)
Shrimp Alkaline Phosphatase (SAP)
Forward or Reverse Sequencing Primer
Distilled Deionized water (ddH_2O)
5x Sequencing Buffer
Big Dye
Betaine (5M)
Sephadex

PCR-compatible 96-well disposable plates
Glass 96-well plates
Collection 96-well plates
An ABI 96-well plate (green) for submission to the CLC.

Procedure

1. *Request an Order Number* from the CLC by following these instructions: http://www.brc.cornell.edu/brcinfo/?p=rtlfaorder.
2. *Perform your desired PCR protocol on your samples* and check their concentration with Agarose gel electrophoresis or with a NanoDrop machine.
    a. Try to have as close to 96 samples (or multiples thereof) as possible, and at least over 48, for cost-efficiency.
    b. Cornell's Life Sciences Core Laboratories Center (CLC), who performs the sequencing service, charges for this service by the plate, not by the sample, so a plate of 10 samples costs our lab the same as a plate with 96 samples.
3. *Dilute PCR products*, if necessary (to get a sequence with more bases).
    a. If bands on gel are weak to moderate, do nothing
    b. If bands on gel are very strong—dilute PCR products by adding ddH_2O (up to 1:1 dilution for very strong bands)
4. *Remove Excess Primers* with Exo-SAP.
    a. To remove excess primers from your PCR products, you need to treat your samples with Exonuclease I (Exo) and Shrimp Alkaline Phosphatase (SAP)
    b. Add 0.3µl of Exo (10 units/µl) and 1µl of SAP (1 unit/µl) to each well of a new plate

- For 1 plate, make a master mix of 33µl Exo and 110µl of SAP and pipette 1.3µl of the master mix into each well.
- Store unused Exo-SAP mix at -20˚C.

c. Add 5µl each of the PCR products to the plate(s), and gently pipette-mix the PCR product with the enzyme mix.
d. Briefly centrifuge the plate(s).
e. Run the samples through the Exo-SAP program on a thermal cycler.

5. *Prepare Sequencing Plate*
   a. Add the following to each well of a new plate (or plates), the Sequencing Plate(s):
      - 2.75 µl ddH_2O.
      - 0.5 µl primer (Forward OR Reverse, NOT both) (10µg/µl).
      - 1.25 µl "Exo-SAP"ed sample.
   b. Briefly centrifuge the plate(s).
   c. Prepare a Master Mix for the Sequencing Plate (multiply volumes below by no. of plates):
      - 440µl ddH_2O
      - 275µl 5X sequencing buffer
      - 55µl betaine (5M)
      - 55µl Big Dye
   d. Add 7.5µl of this master mix to each well of the Sequencing Plate(s) and gently pipette-mix the solution.
   e. Briefly centrifuge the plate(s).
   f. Run the samples on the "RTLseq" program in a thermal cycler.
6. *Purify your product.*
   a. While "RTLseq" is running, prepare Sephadex for each plate in a 50mL tube
      - 40ml H_2O
      - 2.9 grams Sephadex
      - Allow the Sephadex to hydrate for at least 30 minutes.
   b. After "RTLseq" has finished, prepare the purification plate by adding 400µl of the well-mixed Sephadex to each well of a Corning glass plate (use electronic pipette for increased speed and accuracy).
   c. Place the glass plate on an empty collection plate, prepare a balancing plate of equal mass, and centrifuge both for 3 minutes at 2250 RPM.
   d. Add 4µl of ddH_2O to each well of a new ABI plate (green). Write your Order Number (provided to you once you follow instructions in Step 1) on the side of the green ABI plate, as well as any other information for your own reference.
   e. Place glass plate onto the ABI plate and pipette the entire 12µl of sequencing reaction from your Sequencing Plate onto the Sephadex column.
      - IMPORTANT: Do not touch pipette tips to the Sephadex - Hover tips over the Sephadex columns and dispense.
   f. Centrifuge for 3 minutes at 2250 RPM (final volume after spinning should be 16µl).
   g. Seal the plate and bring it, on ice, to the Core Laboratories Center (CLC), Room 147 of the Biotechnology Building, for sequencing.
   h. Clean the glass plate for reuse:
      - Empty Sephadex columns into garbage.
      - Rinse out plate several times.
      - Centrifuge again for 3 minutes at 2250 RPM to remove residual water