The RNAi Consortium

Section II: Preparation of Transfection-Quality DNA for the TRC Library

Introduction:

We have used two methods for 96-well plate-based transfection-grade DNA preparations:

1.) The commercial plate-based PureLink prep from Invitrogen.
2.) A TRC-developed protocol that uses commercial low-grade Whatman prep with a subsequent magnetic bead purification step.

A DNA preparation of pLKO.1 by either of these methods exhibited high transfection efficiencies in 293T cells, comparable to the transfection efficiencies achieved with maxi-prepped DNA. The PureLink method is more expensive but provides more consistent well-to-well yields than the current version of the bead-based protocol.

(1) Commercial DNA Prep Instructions:
We used the Invitrogen PureLink kit according to manufacturers instructions *except* that we grew cells as described in Section III.A and performed an additional heating step described in Section III.C3 (see below).

(2) TRC-Developed DNA Prep Instructions:
We developed a low-cost method using Whatman 96-well filter plates to extract crude DNA followed by Agencourt magnetic particles for purification. This protocol is currently being modified to decrease well-to-well variation in yields. The current Whatman/magnetic bead method is described below.

Whatman/Magnetic Bead DNA Prep and Modifications to Invitrogen PureLink Prep

1. Materials

1. 2.2ml Deep well plate filled with 1.2ml TB (terrific broth) containing 100 ug/ml Ampicillin (Marsh Abgene, Cat# DW 9622).
2. Round-bottom plates (Costar, Corning, Cat# 3795).
3. Gas permeable seals (Marsh BioProduct, Cat# AB-0718).
4. Filter plates (ISC BioExpress, Cat# t-3180-1.)
5. Whatman uniplate receiving plates (Whatman, Cat# 77001800)
6. Agencourt beads (Sera-Mag Magnetic Carboxylate-Modified Particles) (Agencourt, Cat# 4415-2100).
7. 16% PEG in 2M NaCl.
8. P1 (50 mM Tris.HCI, pH8.0, 10 mM EDTA, 100ug/ml RNase A, available from Qiagen).
9. P2 (200 mM NaOH, 1% SDS, may purchase from Qiagen).
10. P3 (3.0 M potassium acetate, pH5.5, may purchase from Qiagen).

II. Equipment

1. Plate Shaker (New Brunkswick, Model: Innova 2300).
2. Hydra 96-well pipettor (Robbins Scientific Corp, Cat# HYDR96 UG RB).
3. Heat sealer (Abgene, Cat# ALPS-300).
4. Jouan centrifuges (Jouan, Model: KR422. Cat# 11178608).
5. Magnetic plate stands (In-house made).
6. Vortex (VWR, Model G-560).

7. Beckman Benchtop Centrifuge (Allegro 6).

III. Instructions

A. GROW CELLS:

1. Inoculate a deep-well containing 1.2ml TB and 100 ug/ml Ampicillin, following protocol from "RNAi Library Handling, Inoculation and Duplication", section B. "INOCULATION & GROWTH".
2. After inoculation, deep-well plates are sealed with gas permeable seals and loaded on plate shaker in 37C warm room.
3. Set shaker at 300rpm. **Deep well plates should grow no longer than 17 hours at 37C.** Over-growing the cells causes low DNA yield.
4. Cells are harvested by centrifuging at 4760 x g for 6min.

B. WHATMAN FILTER DNA PREP:

1. Resuspension:
   -Add 150ul of solution P1 to the deep well plate. Seal plate with Costar Scotch seal.
   -Resuspend cells well by vortexing. Make sure each well is free of cell clumps).
2. Lysing:
   -Add 150ul of solution P2. Re-seal plate with Costar Scotch seal.
   -Mix with vortex on speed 3 for about 5 seconds.
   - Leave the plate at RT for **4 min.**
3. Neutralization:
   - Add 150ul of solution P3. Re-seal plate with Costar Scotch seal.
   - Mix with vortex on speed 3 for about 5 seconds.
   - Put deep well plate on ice or in freezer for 10 minutes.
4. Pellet debris in Jouan centrifuge (25 mins. @ 4000 x g).
5. Fill Whatman receiving plates with 280ul 99% Isopropanol. Put filter plate on top of the receiving plate and tape them together. Label both receiving and filter plates.
6. Transfer supernatant from the just-centrifuged deep well plate into the Whatman filter plate using the Hydra. Make sure to adjust the Hydra tip height so that no cell debris is transferred.
7. Wash the hydra tips between transfers of different plates using dd-H2O.
8. Spin the pair of filter plate and receiving plates in the Jouan centrifuge (5 min @ 1800 x g). This step is to make sure liquid flows through the filter, while residual debris stays at top of the filter.
9. Discard filter and put the receiver plates back in the Jouan centrifuge for precipitation (15min @ 4000 x g). Carefully decant off isopropanol.
10. Add 200ul cold 70% ethanol to receiving plate.
11. Spin using Jouan centrifuge (7 min at 4000 x g).
12. Dump ethanol supernatant. Add 200ul cold 70% ethanol to receiving plate.
13. Spin using Jouan centrifuge (7 min at 4000 x g).
14. Dump ethanol. Air dry on bench top for 10 min.

C. MAGNETIC BEAD (SPRI) CLEAN-UP:

1. After the DNA is dry in the Whatman receiving plate, elute DNA with 50ul de-ionized water. Shake the DNA plate gently for 3 minutes at 600rpm and then briefly spin it in the Beckman centrifuge at 500rpm (~ 47 xg). Transfer 42ul DNA into a 96 costar round bottom plate.
2. Buffer the DNA by adding 5ul of 10x PCR Buffer II (Roche) and 3ul of $MgCl_2$ (25mM).
3. Heat the DNA plate at 70C for 30min using a water bath (Potentially helps by disrupting aggregation).

4. Add 20ul of magnetic beads (Agencourt) to each well. Then add 80uL of hybridization buffer (16% PEG in 2M NaCl) to each well; mix well by shaking the round bottom plate on a single plate shaker for 3 minutes at 600rpm.
5. Let the plate sit at room temperature for 10 minutes.
6. Place the plate on a magnetic stand for 5 minutes. When the supernatant is clear, discard the supernatant by inverting the plate while keeping the plate attached to the magnate.
7. Wash the plate with 150ul EtOH (10mM Tris-Acetate, 95% Ethanol) 3 times. Air dry on bench top for 10 min.
8. Elute the DNA with 50ul of water. Shake the DNA plate gently at room temperature for 30min, or leave it o/n at 4^{o}C. Use the magnetic stand to pull down the beads, so that the clear DNA solution can be transferred to a new plate.