

The RNAi Consortium (TRC)

Broad Institute

TRC Laboratory Protocols

Protocol Title: **Use of Glycerol Stocks and Preparation of Transfection-Quality Plasmid DNA**

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Brief Description:

This protocol contains recommendations for inoculation, growth, and duplication of pLKO (shRNA), pLEX (ORF expression clone), and pEntry bacterial glycerol stocks and a protocol for the preparation of transfection-quality plasmid DNA in 96-well plates.

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Part 1: Inoculation, growth, and duplication of glycerol stocks in 96-well plates

I. Materials and Equipment

TB media (terrific broth; e.g. American Bioanalytical, Cat. AB01965-05000)
Carbenicillin
Autoclaved 50% glycerol
Deep well 96-well sterile growth plates (Corning, Cat. 3960)
Gas-permeable plate seals (Excel Scientific, Cat. B100)
Round-bottom 96-well glycerol storage plates (Corning, Cat. 3795)
96-well HT spectrophotometer (e.g. Bio-tek Synergy)

II. Instructions

A. Storage and handling of glycerol stocks

Hairpin-pLKO, ORF-pLEX and ORF-EntryClone bacterial glycerol stocks should be stored at -80 °C. When using bacterial glycerol stocks, minimize the time thawed.

B. Inoculation and growth in 96-well plates

1. Pre-fill wells in a deep well 96-well sterile growth plate with 1.2 mL TB media containing 100 µg/ml carbenicillin (for hairpin-pLKO or ORF-pLEX clones), or with 1.2 mL LB media constraining 50 µg/ml Spectinomycin (for growing ORF-EntryClone clones). Thaw bacterial glycerol stock(s), mix by pipetting, and transfer 5 µL per well into the deep well growth plate.
2. After inoculation, seal the growth plate with a gas-permeable seal and shake (300 rpm) at 37 °C for at least 16 hours (hairpin-pLKO or ORF-pLEX clones), or 18 hours (ORF-EntryClone clones). If the culture will be used to isolate plasmid DNA, we recommend growing the bacterial culture for 17 hours (hairpin-pLKO or ORF-pLEX clones), 20 hours (ORF-EntryClone clones), or until the OD600 of a 3-fold diluted sample is between 0.4 - 0.6 (see Part 2, Step D).

C. Duplication of glycerol stocks

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Pre-fill wells in round-bottom 96-well glycerol storage plates with 40 μ L autoclaved 50% glycerol. Transfer 80 μ L of culture from the 16 hour deep well growth plate into each destination plate to make replicate copies. Freeze immediately and store at -80 °C.

Part 2: Preparation of Transfection-Quality Plasmid DNA in 96-well Plates

I. Materials and Equipment

Deep well 96-well non-sterile collection blocks (Marsh, Cat. DW9622)
Clarification filter plates (Whatman, Cat. 7770-0062)
pDNA binding plates (Whatman, Cat. 7700-2810)
Plastic plate seals (Qiagen, Cat. 1018104)
96-well DNA storage plates (Corning, Cat. 3790)
Microseal films (BioRad, Cat. MSF1001)
Buffers (see section A below, "Preparation of buffers")
Alkaline protease solution (500 units/ml, Promega, Cat. A1441)
70 °C water bath
Centrifuge (e.g. Jouan KR422, or Jouan CR4i)

II. Instructions

A. Preparation of buffers

1. Resuspension Buffer: 50 mM Tris.HCl, 10 mM EDTA, pH 8.0.
Resuspension Buffer with RNase A: Prior to use, add RNase A for a final concentration of 0.1 mg/ml. Store Resuspension Buffer with RNase A at 4 °C for up to 6 months (as per Qiagen Miniprep Handbook, Ed 2).
2. Lysis Buffer: 200 mM NaOH, 1% SDS (w/v).
3. Neutralization/Binding Buffer: 3.75 M Guanidinium Hydrochloride, 0.9 M KOAc, 1.4 M HOAc, pH 4.35.
4. Wash Buffer: Prepare initial solution of 15 mM NaCl, 40 mM Tris.HCl, 25mM Tris, pH 6.65. To 120 ml of this solution, add 480 ml ethanol (100%) and mix well.
5. Elution Buffer: 10 mM Tris.HCl, pH 8.0.

Note: Buffers may be stored at room temperature for up to 6 months, unless otherwise indicated.

B. Preparation of a fresh copy (V2) of bacterial glycerol stock(s):

1. Follow instructions in Part 1 for inoculation and growth in 96-well plates and duplication of glycerol stocks to make a new glycerol copy (V2 glycerol). These newly prepared V2s are then used in inoculations for plasmid DNA isolation.

Note: Starting with a fresh glycerol copy can increase overall yield and decrease well-to-well variation in yield of transfection-quality DNA.

C. Inoculation and growth of bacterial culture in deep well plates

1. Follow instructions in Part 1 for inoculation and growth in 96-well plates, using the V2 glycerol copy to inoculate.

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2. Grow the bacterial cultures on a plate shaker at 300 rpm, 37 °C for 17 hours, or until the OD600 of a 3-fold diluted sample is between 0.4-0.6 (see step D).

D. Measurement of cell density and harvesting cells

1. Remove 40 µL of each bacterial culture and dilute with 80 µL fresh media. Measure the optical density of the diluted samples at 600nm (OD600). If the OD600 of the diluted samples are between 0.4 - 0.6, proceed with harvesting cells. If the OD600 measurements are less than 0.4 for a significant number of wells, continue to grow the bacterial cultures until the OD600 of 3-fold diluted samples are between 0.4 - 0.6.
2. Centrifuge the growth plate at 1,500 x g for 8 minutes at 4 °C.
3. Remove the TB or LB media by inverting the growth plate. Gently tap the inverted plate against a paper towel to remove as much liquid as possible without disturbing the bacterial pellets.

E. Purification of plasmid DNA (centrifugation method)

1. Add 200 µL Resuspension Buffer with RNase A to each well of the growth plate, seal the plate, and vortex for several minutes until the bacterial pellets are fully resuspended.
Note: in steps 1 - 6, seal the plate with a fresh plastic seal as indicated.
2. Add 210 µL fresh Lysis Buffer to each well, seal, and gently tilt the plate back and forth a few times. Incubate at room temperature for exactly 4 minutes and proceed immediately to the next step.
Note: This is a time sensitive step.
3. Add 300µL Neutralization/Binding Buffer to each well, seal, and mix by tilting the plate back and forth a few times. Centrifuge at 3,000 x g for 30 minutes at 4 °C.
4. Place a clarification filter plate on top of a deep well collection block. Use a multichannel pipette to transfer the clear liquid lysate from each well of the growth plate into the clarification filter plate as described below.

Note: Try to avoid transferring cell debris from the bottom of the wells to the clarification filter plate, especially in the first transfer step.
 - a. Transfer 500 µl lysate to the clarification filter plate by pipetting from the top of the well to obtain only clear liquid. Retain the remaining lysate at 4 °C.
 - b. Centrifuge the stacked clarification filter and collection block at 3,000 x g for 5 minutes at 4 °C.
 - c. Keep the clarification filter and collection block stacked together and transfer the remaining lysate (~175 µl, may contain some cell debris) to the clarification filter plate.
 - d. Centrifuge again at 3,000 x g for 5 minutes at 4 °C. Discard the clarification filter plate and retain the collection block.
5. Stack a pDNA binding plate on top of a clean collection block. Transfer the entire contents of the collection block from step 6 to the pDNA binding plate. Discard the used collection block.
6. Centrifuge at 1,800 x g for 2 minutes at 4 °C. Discard the flow-through from the collection block.
7. Place the pDNA binding plate back on the collection block. Add 600 µL Wash Buffer to each well of the pDNA binding plate. Centrifuge at 1,800 x g for 2 minutes at 4 °C. Discard the flow-through from the collection block.

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8. Repeat step 8 for a second wash.
9. Place the pDNA binding plate back on the collection block. Centrifuge at 1,800 x g for 5 minutes at 4 °C to dry the pDNA binding plate.
10. Place the pDNA binding plate on a clean DNA Storage Plate. Add 140 µL Elution Buffer to each well of the pDNA binding plate and incubate for 10 minutes at room temperature. Centrifuge at 1,800 x g for 5 minutes at 4 °C. Expected elution volume: 120-130 µL DNA per well.
11. Seal the DNA Storage Plate with a Bio-Rad microseal film and store at -20 °C or -80 °C.
Note: Use a plate seal that can withstand the storage conditions.

Version Notes:

1/22/07

- 1) **Innoculation with fresh glycerol copy (V2) of hairpin-pLKO, ORF-pLX or ORF-EntryClone bacterial glycerol stock(s):** Using a fresh bacterial glycerol stock to inoculate growth increases both overall yield and well-to-well yield consistency of pLKO.1 plasmid DNA.
- 2) **Alkaline Protease added to the lysis buffer:** Adding Alkaline Protease to the Lysis Buffer improves the yield and quality of plasmid DNA. See <http://www.promega.com/faq/wizsv.html> for more information.

4/1/09

- 3) **Discontinued using Alkaline Protease in the lysis buffer:** We experienced Alkaline Protease (AP) quality issues that caused significant DNA prep fails and consumed a great deal of time in troubleshooting. The drawback of the inconsistent AP quality outweighed the advantages AP can offer.
- 4) **Discontinued 70 °C (30 minutes) incubation prior to binding DNA to the binding filter:** We determined that this is an unnecessary step.