Vanderbilt Microarray Shared Resource

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PCR Amplification Protocol for the NIA 15K set

Introduction:

Amplification of the cDNA clones represented in the 15k NIA clone set is a challenge. To effectively produce microarrays from these clones, the amplifications should be efficient, low cost, and reproducible with a low rate of cross contamination. To this end, we have used the protocols developed by TIGR (Hedge,*et al*. Biotechniques 29:548-562. Sept. 2000) with a few minor modifications.

Materials:

- Fresh overnight cultures grown in 96-well plates. We have found that as cultures 'age' when stored at 4 ºC, the uniform loading of replicator slot pins is affected.
- 1µl or 5µl slot pin replicators (*V & P Scientific. Vp408S5, VP408S10 www.vp-scientific.com)*
- Primers: (These were developed by TIGR and can be used on most vectors which have M13 sequences)
  M13 FWD: **5'-GTTTTCCCAGTCACGACGTTG-3'**
  M13 REV: **5'-TGAGCGGATAACAATTTCACACAG-3'**
- Standard PCR Reagents
- 96-well PCR plates (*Fisher 05-500-63*)
- 96-well u-bottom plates(*Costar 3795*)
- Thermal cycler (*MJ Research PTC-2225-we have 3 tetrads and typically run 12-96-well PCR plates at a time*)
- Microseal A Mats for sealing PCR plates (*MJ Research MSA 5001*)

Methods:

DAY 1:

INOCULATION

- 165µl of LB containing 100µg/ml AMP is dispensed using a Labsystems Multidrop DW into 12 96-well U-bottom plates(*Costar 3795*).

- A 1 µl slot pin replicator(*V&P Scientific*) is used to inoculate each 96well plate, and the plate is covered with Qiagen Air Pore tape sheets*(#19571*) and incubated overnight at 37°C with shaking for 16 – 18 hours.

DAY 2:

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LYSIS

- 50µl of milliQ H2O is dispensed into 96-well PCR plates (*Fisher*) and placed on ice (to reduce loss of volume from evaporation) using a Labsystems Multidrop DW.
- Overnight cultures are inoculated into the PCR plates containing MilliQ water using a 10µl slot pin replicator *(Note: we have found that we needed a larger inoculum in the lysis step than that recommended by TIGR.)*
- The PCR plates are covered with a thermal seal (MJ Research-Microseal A film MSA 5001) and incubated at 95°C for 10 minutes.
- Cellular debris from the lysed cells are pelleted by centrifugation at 1200xg for 4 minutes in a centrifuge equipped with microplate carriers.

PCR

- 20µl of MilliQ H2O is dispensed into 96well PCR plates and placed on ice (to reduce loss of volume from evaporation) using a Labsystems Multidrop DW.
- A 5µl slot pin replicator is used to transfer approx. 5–10µl (2x inoculation of the 5µl or a single inoculation with the 10µl slot pin replicator) of the lysate into the 20µl of Milli-Q water and the inoculum is placed on ice *(Note: lysates may be prepared in advance and stored at –20°C).*
- A Master mix is made for the 12- 96 well plates as follows (each well houses one reaction):

Per Reaction:	
10X PCR buffer	11µl
dNTP's	0.9µl
(M13F/M13R)	0.256µl (20pmoles/each primer per reaction)
Taq	5 Units/rxn
H2O(milli-Q)	76 µl
	90µl total

Notes:
Stock dNTPs are 25 mM each dNTP.
Primer concentration is 20 pmol of each primer/reaction.
Units/reaction of Taq required will vary by manufacturer. We have had good success with Taq from Promega as well as Stratagene (Yieldace).
If the Lab Drop dispenser is used, add a 1.5 plate dead volume due to losses in the tubing.
Multiply the per reaction volumes by the number of reactions planned + 2 reactions to account for pipette variation.

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- 90µl of master mix is added to each well containing the bacterial lysate to give a final reaction volume of 110µl /well using the Labsystems Multidrop.

- The PCR plates are covered with microseal A (*MJ Research*) and amplified by using the following conditions:

  - Initial denaturation step of 3 minutes at 96°C
  - 40 cycles: 95°C 30 sec
    55°C 30 sec
    72°C 2.5 minutes (product sizes range up to 3kb)
  - Final extension for 7 minutes at 72°C
  - Soak at 4°C

- PCR products are analyzed by agarose gel electrophoresis. We routinely run out 5µl of product on 1.5% agarose gels to check for yield and multiple products. PCR reactions are quantitated based on molecular mass markers (*BIORAD EZ Load Precision Molecular Mass Ruler*). Yields ranged from 400 ng to 20 µg/rxn with a median yield of 10 µg. Gel images from all 15,000 clones in the NIA set can be found on the VMSR website:
  http://array.mc.vanderbilt.edu/Images/PCR_Images/NIA_PCR_Images/nia_pcr_images.html

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