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1. PURPOSE

This protocol describes PCR amplification of eukaryotic cDNA plasmid inserts, gel electrophoresis, purification, and storage of PCR products.

2. SCOPE

This procedural format is currently utilized by Human Colon Cancer and Mouse microarray projects under the supervision of John Quackenbush within the Eukaryotic Genomics Dept.

3. MATERIALS

3.1 AmpliTaq® DNA Polymerase with GeneAmp® PCR Reaction Kit (Applied Biosystems; Cat # N808-0156)
-10X PCR Buffer II
-$MgCl_2$ Solution
-Platinum Taq Polymerase
3.2 M13 Forward and Reverse Primers (Life Technologies)
- Forward: 5' GTT TTC CCA GTC ACG ACG TTG 3'
- Reverse: 5' TGA GCG GAT AAC AAT TTC ACA CAG 3'
3.3 dNTP kit (100mM of each dNTP) (Life Technologies; Cat # 10297-018)
3.4 MilliQ water
3.5 Hard-Shell Skirted 96 well Microplate (MJ Research; Cat# HSP-9601)
3.6 MicroAmp® 96-well Full Plate Cover (Applied Biosystems; Cat # N801-0550)
3.7 Multiscreen® PCR filter plate (Millipore; Cat # MANU3050)
3.8 Millipore Filtration Manifold (Millipore; Cat # MAVM0960R)
3.9 Cap mat (VWR; Cat # 40002-002)
3.10 Falcon Microtest U-bottom 96 well plate (BD Biosciences; Cat # 353077
3.11 Electrophoresis Gel Box Kit--Casting/running trays, combs, wires (CBS Scientific; SGE-1215-Red)
3.12 Agarose
3.13 Ethidium bromide (EtBr)
3.14 1 Kb DNA ladder (1.0 μg/μL) (Life Technologies; Cat# 15615-016)

4. PROCEDURE

4.1 PCR of cDNA plasmid inserts.

4.1.1 After cDNA clones have been cultured and plasmids isolated (see SOP- M001), a 1:10 dilution is made of the concentrated plasmid isolate (10 µL in 90 µL of MilliQ H_2O).

4.1.2 Prepare a master mix for 100 µL PCR reactions according to the following mixture:

MilliQ water……	7.32 mL
10X PCR Buffer……..……..	1 mL
$MgCl_2$ (25 mM)……...……..	1 mL
M13 Forward primer (10µM)…….	200 µL
M13 Reverse primer (10µM)……..	200 µL
dNTP mix (25mM per dNTP)*…..	80 µL
(* 20 µL of each dNTP as 100 mM stock)	
Platinum Taq (5U/µL)…….	40 µL
Total Volume/Plate	9.84 mL

Note: Keep all reagents on ice while preparing the master mix. Add the Platinum Taq Polymerase last taking it directly from the freezer and returning it promptly.

Note: Prior to preparing PCR sterilize work space, wear gloves and always use sterile tips to avoid contamination.

4.1.3 Mix master mix well and decant into a sterile reservoir. Using an automatic multichannel pipette (8 channel Matrix Impact^2) dispense 96 µL of mix into each well of a Hard-Shell Skirted 96 well Microplate.

4.1.4 Add 4 µL of 1:10 diluted plasmid (from step 4.1.1) to each well containing master mix. Mix well with the pipette.

4.1.5 Place a MicroAmp® Full Plate Cover on the PCR plate and centrifuge at 2700 rpm for 1 minute.

4.1.6 Place PCR plates in a thermocyler (MJ Research; PTC-225) and run the following cycling program:

Initial Denaturation……	95°C x 2 min.

Denaturation……..95°C x 30 sec.
Primer annealing……52°C x 30 sec.
Primer extension……72°C x 2 min.
} x 30 cycles
4°C forever

4.1.7 Centrifuge, seal, and store PCR reaction plates at 4°C or frozen at –20°C.

4.2 Preparation of a 1% agarose gel for 96 PCR samples.

4.2.1 Prepare ethidium bromide (EtBr) stock solution (10mg/mL) with water and store at room temperature in a dark bottle.

Caution: Ethidium bromide is a powerful mutagen and toxin. Gloves and eye protection should be worn when handling gels or solutions containing EtBr. EtBr waste should be contained and disposed of according to institutional policy.

4.2.2 Prepare a 1% agarose solution (1.1g agarose/ 110mL 1X TBE for one gel) and microwave until agarose is well dissolved.

4.2.3 To the dissolved agarose add 5 µL of ethidium bromide stock solution (10mg/mL) and swirl to mix. Final concentration should be approx. 0.5 µg/mL.

Note: Melted agarose can be covered and kept in a 60-65°C water bath until needed.

4.2.4 Place gel combs in the casting block slots and securely place the casting block in a level holding tray.

4.2.5 Carefully pour the melted agarose solution (~110mL) to the center of the casting block. Remove any bubbles that may form during pouring.

4.2.6 After gel has sufficiently cooled and solidified carefully remove combs. Without tearing the gel remove the casting block/gel from the holding tray.

4.2.7 Place casting block/gel in an electrophoresis chamber containing 1X TBE buffer. The gel should be submerged so that it is completely covered with ~1mm of buffer.

4.3 Loading and running gels

4.3.1 Prepare 2X gel loading buffer with 0.25% bromophenol blue and 50% glycerol in water.

4.3.2 In a 96 well Microtiter plate add 5 µL of 2X gel loading buffer. Transfer 5 µL of each PCR product from the PCR reaction plate to the Microtiter plate containing loading buffer. Mix well.

Note: 1 Kb ladder mix:

400 µL ddH_2O
100 µL 1 Kb DNA Ladder (1.0 µg/µL)
+ 500 µL 2X gel loading buffer
1.0 mL 1 Kb ladder Mix (0.1 µg/µL)

4.3.3 In each gel are four rows containing 26 wells each. Each row of the gel will have 1 Kb DNA ladder mix in the left most well (5 µL) followed by 24 PCR samples (the last well will be empty). With a twelve channel pipette add the 10 µL PCR samples from row A (96 well plate) into wells 2,4,6 etc. from left. Add samples from row B to wells 3,5,7 etc. Likewise samples from rows C and D will be added to the second gel row, E and F added to the third and G and H to the fourth.

Note: Ethidium Bromide, being positively charged, has a tendency to migrate out of the gel and toward the negative terminal; thus the bottom of the gel may be dim when exposed to UV light. To overcome this effect, one can add 5 µL of EtBr to the buffer reservoir closest to the positive terminal prior to applying current.

4.3.4 Once all samples have been loaded, cover gel box and connect power supply cables (+ cable downstream, - cable upstream).

4.3.5 Apply ~100 volts of power for approx. 45-60 minutes.

4.3.6 Remove the gel tray from the gel box; slide the gel out of the casting block and onto the imaging platform.

4.3.7 After capturing the gel image discard the gel in a waste container designated for EtBr waste.

4.3.8 The 1X TBE gel buffer can be reused to run 10-12 gels before being replaced. If EtBr has been added to the buffer make sure it is handled and disposed of as EtBr waste.

4.4 Purification of PCR product

4.4.1 Spin down PCR reaction plates and then transfer the PCR products (100µL) to a Multiscreen® filter plate and place the filter on a vacuum filtration manifold (Millipore; Cat # MAVM0960R).

4.4.2 Apply a vacuum pressure of approx. 10-15 inHg (250-380 mmHg) for ten minutes or until plate is dry.

Note: Filter until no more fluid is visible in the well. The filter appears wet (shiny) even when dry so do not use the appearance of the filter as a guide. Also check all of the wells in the plate before removing from vacuum; some wells filter more slowly than others.

4.4.3 Remove plate from filtration manifold and add 100 µL of MilliQ water to each well (for more concentrated PCR products resuspend in less volume).

4.4.4 Place filter plate on a shaker and shake vigorously for 20 minutes to resuspend the DNA.

4.4.5 Manually pipette the purified PCR product to a new Falcon U-bottom 96 well plate.

4.5 Storage of PCR products

4.5.1 Seal PCR storage plates with a plastic cap mat or adhesive foil lid and store at –20°C until needed for making microarray printing plates.

4.5.2 For long term storage after filtration aliquot purified PCR product into multiple plates. Store one plate with a cap mat at –20^{o}C (for short term use) and dry down the remaining plates with a speed vac and store in a dry cabinet.

4.5.3 To make printing plates, resuspend dried PCR product with MilliQ water and refer to SOP: M003 for further instructions.