Amplification of Mouse cDNAs for Microarrays Using the Eppendorf MasterTaq Kit

Katrine Verdun, Richard Rouse, and Gary Hardiman

BIOGEM,
Division of Biology,
University of California San Diego,
La Jolla, CA 92093-0349
e-mail: ghardiman@ucsd.edu
www.biogem.ucsd.edu

Introduction

Microarray technology, primarily advanced by Pat Brown and his colleagues at Stanford University, makes use of a robotic spotting device or "microarrayer" to spot DNA sequences onto "derivatized" glass slides (1). These arrays are then hybridized with cDNA probes and analyzed using methods conceptually similar to those described for Affymetrix® gene chips (2). Since the cost of producing an individual array is relatively small, spotted arrays are highly versatile and can make use of a wide variety of clone sets. Microarrays thus enable individual investigators to perform large-scale analysis of model organisms or to customize arrays for special genome applications.

The cDNAs spotted on microarrays are typically PCR products, amplified by PCR from plasmid miniprep DNA of cDNA clones. The recommended concentration for spotting on aminosilane and polylysine slides is 200-400 fmol/µl. Lower concentrations than this will result in poor signals and higher concentrations are not recommended as target spots are seen to smear. Microarray hybridizations work poorly with weak PCR products. Consequently, PCR amplification is optimized to maximize DNA yield. In this report we describe the use of the Eppendorf® MasterTaq® Kit in the preparation of targets for a mouse ~8 K microarray.

Materials and Methods

- *PCR amplification*

Selected clones were inoculated into a 96-well Falcon® U-bottom plate (Nunc) containing LB/Ampicillin (50 mg/ml) and were placed in a shaking incubator (200 rpm) overnight at 37 °C. PCR reactions (50 µl) were assembled using a liquid handling workstation and consisted of 10 µM dNTP mix, 20 µM primer mix (forward primer 5'-ctg caa ggc gat taa gtt ggg taa c-3' and reverse primer 5'-gtg agc gga taa caa ttt cac aca gga aac agc-3') and 1 µl of overnight culture. Amplifications were carried out using 7.5 Units of polymerase in the recommended buffer, in accordance with the instructions provided by the manufacturer. Thermocycling was performed using the following cycling conditions: an initial five-minute denaturation at 95 °C to lyse the cells and release the plasmid DNA, 35 cycles (94 °C for 1 minute, 58 °C for 1 minute, 72 °C for 4 minutes), followed by 10 minutes at 72 °C. Conditions recommended by manufacturers of Hot Start polymerases typically include a 15-minute rather than a 5-minute initial denaturation step. One-fifth of the PCR amplification was analyzed on a 0.8% agarose gel containing ethidium bromide and was visualized with an AlphaImager® 2200 Documentation and Analysis system.

The mouse GEM 1 Clone List (build 35) containing 8,734 clones was purchased from Incyte® Pharmaceuticals (Palo Alto, CA). For amplification of this mouse Unigene® set, reactions were carried out in a 96-well plate format using 3 µl of overnight culture and either 7.5 or 3.75 U MasterTaq respectively, in a final volume of 150 µl. Successful PCR amplifications were verified by electrophoresis on agarose gels. PCR products were purified using a commercially available kit. The yield of PCR amplicon was assayed using the Picogreen® dsDNA quantitation kit (Molecular Probes) and a Bio-Tek® FLX800 microplate fluorescence reader.

- *Preparation of fluorescently labeled cDNA*

One µg of polyA+ RNA (mouse spleen and liver, respectively) were converted into fluorescently labeled cDNA by incubation with an oligo dT primer (44 µM final) at 70 °C for 10 minutes in a final volume of 9 µl. The solution was then mixed with 11 µl of a solution 1.8X first strand buffer, containing 18 mM DTT, 900 µM dATP, 900 µM dGTP, 900 µM dTTP, 230 µM dCTP, 230 µM Cy3- or Cy5-dCTP

(APBiotech™, UK), 20 U of RNase Inhibitor and 200 U of reverse transcriptase. The reaction was incubated at 42 °C for 2 hours and heated at 100 °C for 5 minutes. RNA was hydrolyzed by the addition of 1 µl of 10 N NaOH and subsequent incubation at 37 °C for 15 minutes. The reaction was neutralized by the addition of 5 µl of 1M Tris pH 7.2 and 2 µl of 5N HCl, and cDNA probes were purified using a commercially available purification kit. Typically, one-third of each reaction was used per hybridization.

- *Microarray fabrication, hybridization, washing, scanning, and data analysis*

PCR products (1,152 in total) derived from the mouse Unigene set and control cDNA clones were printed on a reflective slide (APBiotech, UK). DNA clones were heat denatured for 3 minutes at 95 °C, chilled on ice, and arrayed using a Molecular Dynamics® (Sunnyvale, CA) Generation III spotter. After printing, the microarrays were allowed to dry completely at ambient conditions. The slides were pretreated with 2x SSPE, 0.2% SDS at 55 °C. The cDNA probe was lyophilized and redissolved in ~32 µl Microarray Hybridization Buffer Version 2 (APBiotech, UK). The solution was added to the microarray and a coverslip was applied. Hybridization was allowed to proceed for 14-18 hours at 42 °C. The microarray was washed with 1X SSC/0.2% SDS for 5 minutes at 45 °C, followed by two 5-minute washes with 0.1X SSC/0.2% SDS at room temperature. The microarray was rinsed briefly with water, dried with nitrogen, and scanned using a Molecular Dynamics Generation III scanner.

Results and Discussion

For the analysis of gene expression in eukaryotes, expressed sequence tag (EST) data represent an invaluable resource for gene identification. EST's are single pass partial cDNA sequences. In general, cDNA clones are selected to represent as many unique transcripts as possible. One such method to group transcripts is the Unigene clustering of data sets which attempts to identify unique human transcripts within EST data (http://www.ncbi.nlm.nih.gov/UniGene). In amplifying EST collections for the production of microarrays, the yield of each PCR amplicon is a very important consideration. The optimal concentration of DNA for successful microarray experimentation is typically 200 µg/ml. Hybridizations using non-covalent attachment chemistries (e.g. polylysine, aminosilane) generally work poorly with DNA spotted at concentrations lower than this. When amplifying thousands of clones, the main objective is to minimize the total number of reactions needed to generate sufficient material for printing. A high yield of PCR product includes plentiful amounts of DNA available for spotting, a reduction in cost and labor, and ultimately the production of greater numbers of arrays per PCR reaction.

- *Comparison of the efficiency of amplification of thermostable polymerases from bacterial cultures*

Eppendorf MasterTaq and ten other polymerases were evaluated for the ability to amplify directly from overnight bacterial cultures. Approximately 1 µl of the overnight culture was used to evaluate each enzyme in a reaction volume of 50 µl. The main criteria for evaluation of the polymerases were specific amplification and maximum yield of the desired PCR product (Fig. 1).

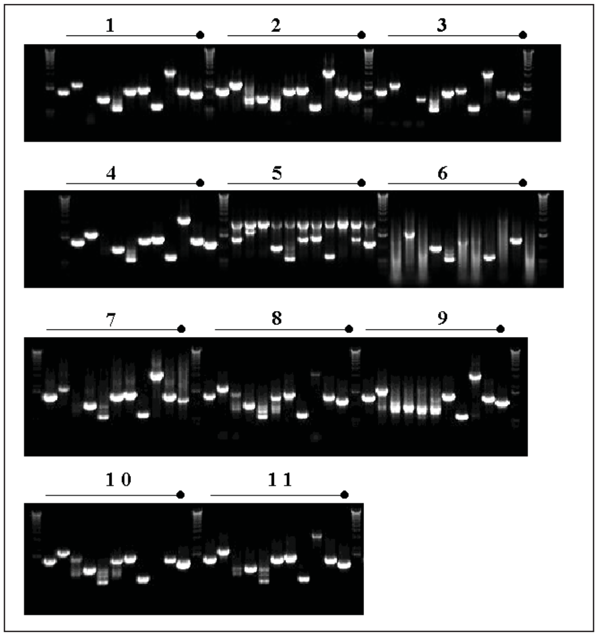

Figure 1: The Eppendorf MasterTaq (#2) and ten other polymerases were evaluated for the ability to amplify directly from overnight bacterial cultures.

Polymerases:

1. Eppendorf *Taq*	4.Takara®	8. Platinum
2. Eppendorf MasterTaq	5. Home Brew	9. Platinum Hi-Fi
3. Amersham® *Taq* Polymerase	6. Amplitaq Gold®	10. Stratagene® *Taq* 2000
	7. Qiagen Hot Start	11. Stratagene Herculase

Upon comparison of ten enzymes, it was observed that enzymes 5 and 6 failed to generate specific products. Enzymes 1, 2, 3, 4, and 7 all performed well in terms of yield of PCR product and absence of non-specific amplicons, but enzyme 2 had no failures. The use of enzymes 8, 9, 10, and 11 all resulted in the amplification of extraneous bands. Given that enzyme 2 (Eppendorf MasterTaq) amplified all targets specifically, it was then investigated as to whether or not a dilution of the enzyme would still result in successful amplification from an overnight culture.

The enzyme was tested at full strength (7.5 U/150 µl reaction) and at half strength (3.25 U/150 µl reaction). We observed that a one-half strength dilution of the enzyme yielded successful amplification of DNA from bacterial clones (Fig. 2).

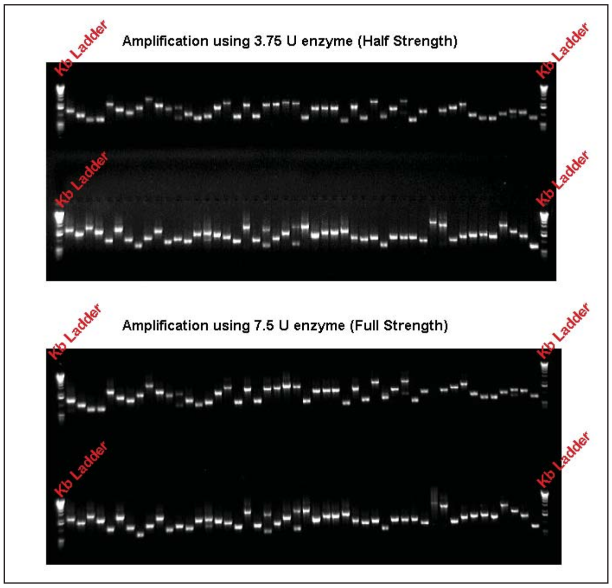

Figure 2: Comparison of Eppendorf MasterTaq at full- and half-strength concentrations in amplifying overnight cultures, 96-well plate amplification of mouse clones.

Amplification of the mouse Unigene set using the Eppendorf MasterTaq Kit

The Eppendorf MasterTaq Kit was used to amplify all ~8 K targets that comprise the mouse Unigene set. In total 8,734 amplifications were performed. Figure 2 shows agarose gel analyses of representative mouse cDNA amplifications using the Eppendorf MasterTaq Kit in a 96-well plate format. In general, strong unique amplicons were observed. The results were classified as follows:

- ~ 7,997 single amplicons (strong bands)
- ~ 359 single amplicons (weak bands)
- ~ 293 multiple bands (typically two or more)
- ~ 85 failures

The cDNA clones that generated multiple bands were re-racked and amplified with a nested primer set (data not shown). For the most part, multiple bands were still observed indicating that these cDNAs represent mixed clones. This signifies ~3.4% contamination, typically found in amplification of a large clone set.

Source Plate Name	Avg. Total ng/Plate
4W-6Z	5541.5
7W-9Z	5367.9
10W-12Z	5174.5
13W-15Z	4848.7
16W-18Z	5187.3
19W-21Z	4169.5
22W-6Yamm.	4401.6
Avg. Total ng in Mouse EST	4955.9

Table 1: Yield of DNA from amplification of the mouse Unigene set. Eight samples from 96-well plates containing purified DNA were assayed for DNA concentration. The plates assayed were numbered 4W, 4X, 4Y, 4Z, 5W, etc. through 22Z. There were five additional plates called 24W, 24X, 24Z, 23W, and 6Yamm respectively.

Quantitation of amplicon yield

All of the PCR products were purified on a liquid handling workstation and with a commercially available kit. Representative DNA samples from the entire set of amplifications were then quantitated using the Picogreen kit (Molecular Probes) to determine an estimate of the yield of purified PCR product. The results are shown in Table 1. The mean clone total yield was 4955.9 ng. The DNAs were resuspended at a concentration of ~200 µg/ml in 25 µl 50% DMSO-H_20. Many microarray spotters use 200 nl per print run and have a void volume of ~5 µl. Assuming the spotter can print 36 arrays per run, this equates in theory to approximately 3,600 microarray slides. The working number of slides however is lower than this due to sample evaporation and other printing variables.

Screening of a mouse microarray with cDNA probes generated from mouse liver and spleen polyA+ RNAs

The final experiment performed was to verify that the PCR products amplified using the Eppendorf MasterTaq Kit generated strong specific signals in a microarray experiment. We printed 1,152 PCR products representing mouse cDNA clones and appropriate controls on a reflective slide (APBiotech, UK). Fluorescent probes were synthesized from mouse spleen and polyA+ RNAs and were used to screen the microarray (Fig. 3). Green, red, and yellow spots were observed, indicating positive hybridization of the labeled probes to the printed PCR products. The enlarged area shows the signal color range observed when using cyanine probes, Cy3 spleen, and Cy5 liver. Green (Cy3) and red (Cy5) spots indicate differential expression of these particular mRNAs sequences in spleen and liver respectively, whereas yellow spots indicate similar levels of expression in both tissues.

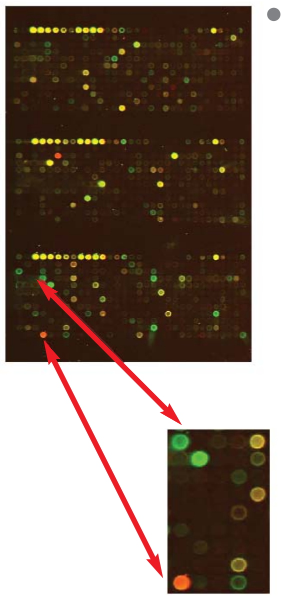

Figure 3: *1,152 cDNA clones derived from the mouse Unigene set and controls were amplified with the Eppendorf MasterTaq Kit and printed on derivatized glass slides. Fluorescent probes were synthesized from spleen and polyA+ RNAs and used to screen the microarray.*

The enlarged area shows signal color range when using cyanine probes, Cye3 spleen and Cye5 liver. Green, red, and yellow spots indicate positive hybridization from the labeled probes. Green (Cye3) and red (Cye5) spots indicate that the targets are expressed differentially.

Conclusion

For generation of PCR amplicons for microarray analyses, the *Taq* from the Eppendorf MasterTaq Kit performs exceptionally well, even when used at one-half of the recommended concentration. High yields of specific amplicons are obtained which can be used to generate high quality microarrays.

References

1. Schena, M.; Shalon, D.; Davis, R.W.; and Brown, P.O. (1995). Quantitative monitoring of gene expression patterns with complementary DNA microarray. Science 270: 467-470.
2. Lockhart, D.J.; Dong, H.; Byrne, M.C.; Follettie, M.T.; Gallo, M.V.; Chee, M.S.; Mittmann, M.; Wang, C.; Kobayashi, M.; Horton, H.; and Brown, E.L. (1996). Expression monitoring by hybridization to high-density oligonucleotide arrays. Nature Biotechnol. 14: 1675-1680.

Ordering Information

Description	Catalog No.
MasterTaq Kit (100 U)	954 14 003-2
MasterTaq Kit (250 U)	954 14 004-1
MasterTaq Kit (500 U)	954 14 008-3
MasterTaq Kit (1,000 U)	954 14 009-1