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**Bert Gold, Julie Bergeron,
Marcia Lachtermacher-
Triunfol, and Michael Dean**
*The National Cancer Institute
at Frederick
Frederick, MD, USA*

Optimized Growth Conditions for Direct Amplification of cDNA Clone Inserts from Culture

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Microarray technology plays a significant role in today's quest for gene discovery and expression. DNA mi-

Table 1. Reaction Master Mixture Prepared for Each 96-Well Microplate

Milli-Q® water	3.2 mL	
10× PCR buffer	500 µL	
MgCl ₂ (25 mM)	500 µL	
M13 forward primer (10 µM)	100 µL	
M13 reverse primer (10 µM)	100 µL	
dNTP mixture (2 mM/dNTP) ^a	500 µL	(Life Technologies, Rockville, MD, USA)
Platinum Taq (5 U/µL)	20 µL	(Life Technologies)
Total:	4.92 mL	
In the mixture, the M13 amplification primers are M13 forward primer, 5'-GTTTTCCTCCAGT-CACGACGTTG-3' and M13 reverse primer, 5'-TGAGCGGATAACAATTCACACAG-3'.		
^a 10 µL each dNTP as 100 mM stock in 460 µL Milli-Q water.		

croarrays are typically constructed by arraying a large number of PCR products on derivatized glass slides. Gene expression is then assayed in a co-hybridization assay that allows two distinct tissue types, developmental stages, or cellular phenotypes to be compared (2). High-quality, high-yield PCR products are a crucial element in obtaining accurate, consistent, and reliable results from microarray assays. PCR products can be prepared using either purified plasmid templates or directly from bacterial culture; many groups preparing microarrays have opted for the second approach because it avoids the cost and labor associated with plasmid template preparation (1). To date, we have amplified more than 30 000 cDNA clones from bacterial culture for printing microarrays. We observed that the manner in which the cultures are handled before PCR amplification has a significant impact on both the quality and quantity of the products. For re-arrayed libraries used for microarray construction, it is often difficult to obtain uniform growth from frozen glycerol stocks and consequently difficult to achieve uniform PCR amplification. To address this problem, we investigated a variety of growth conditions and optimized these for direct PCR amplification from bacterial culture.

For each growth method, clone inserts were amplified in 50 µL reactions in 96-well microplates (Applied Biosystems, Foster City, CA, USA) using an identical protocol. Table 1 shows the master mixture that was prepared for each 96-well microplate.

For each clone, 48 µL master mixture is added to 2 µL supernatant prepared from bacterial culture. Reactions are amplified in a thermocycler (PTC-225 Tetrad™; MJ Research, Waltham, MA, USA) using the following cycling protocol: initial denaturation at 95°C for 2 min, 30 cycles each of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 2 min, and hold at 4°C.

Bacterial supernatant was prepared from culture, starting with -80°C glycerol stocks, using each of the methods described below. PCR amplification products were checked by agarose gel electrophoresis in a 1.5% gel, stained with ethidium bromide, and visualized by UV transillumination.

In Method 1 (Figure 1A), 5 µL cells from the -80°C glycerol stock plate were added directly to 95 µL double-distilled water in a 96-well, U-bottom microplate (Falcon®). The microplate was then placed in a 95°C laboratory oven for 10 min to lyse the cells and release the plasmids. Following lysis, the microplate was centrifuged at 1200× g for 3 min. The supernatant (2 µL) was then added to the 96-well microplate containing the PCR master mixture and amplified as previously described.

In Method 2A (Figure 1B), 5 µL cells from the -80°C stock plate were inoculated into a 96-well, deep-well block (Qiagen, Valencia, CA, USA) with each well containing 1.25 mL LB/ampicillin (50 µg/µL). The cells were grown for 16 h in a 37°C shaking incubator at 200 rpm. Following overnight growth, 5 µL culture from each well

Benchmarks

Figure 1. Representative PCR products generated using each of the three methods: (A) direct from frozen stocks, (B) 16-h overnight growth, and (C) pre-inoculation growth followed by a 16-h overnight growth. For each condition, identical clones from two 96-well microplates were grown and amplified, and equal quantities were run on a 1% agarose ethidium bromide gel. As can be easily seen from these images, method 3 provides much more uniform, higher-yield amplification. Results from method 2B, which involves a 20-h overnight growth rather than 16 h, are not shown because they are not significantly different from those in method B.

were added to 95 μ L double-distilled water in a 96-well, U-bottom microplate. The cells were lysed as in Method 1, centrifuged at 1200 $\times$ g to remove cellular debris, and 2 μ L supernatant from each clone was used for PCR.

In Method 2B (data not shown), clones were handled exactly as in Method 2A, except that clones were grown for 20 h instead of 16 h.

For Method 3 (Figure 1C), 5 μ L cells were inoculated from a -80°C glycerol stock plate into 150 μ L LB/ampicillin (50 μ g/ μ L) in a 96-well, U-bottom microplate. The cells were grown at 37°C in a non-shaking incubator for 7–9 h, until either very light, uniform pelleting had occurred or all of the wells had growth. From this culture, 5 μ L was then inoculated into 1.25 mL LB media/ampicillin (50 μ g/ μ L) in a 96-well, deep-well block. The cells were grown for 16 h in a 37°C shaking incubator at 200 rpm. Following overnight growth, 5 μ L culture from each well were added to 95 μ L double-distilled water in a 96-well, U-bottom microplate. The cells were lysed as in Method 1, centrifuged at 1200 $\times$ g to remove cellular debris, and 2 μ L supernatant from each clone were used for PCR.

Clone inserts were amplified from bacterial culture prepared using the protocols described above, and the reaction products were evaluated by gel electrophoresis on ethidium bromide-stained agarose gels as shown in Figure 1. As can be seen, different growth con-

ditions produce very different amplification results. The performance of Method 1, in which the amplification was done directly from frozen glycerol stock, was clearly inferior to the other methods in which the bacteria were grown before amplification. This suggests that some period of growth is essential for good amplification.

A comparison of the results from Methods 2A and 2B, suggests that growth time also has a significant impact on the amplification results (data not shown). For periods longer than 16 h, amplification efficiency decreases, possibly because the cells are no longer in log phase but have reached stationary phase and started to die; these unhealthy cells do not amplify as well as healthy cells.

However, even with optimal growth times, not all clones grow equally well from frozen stocks. In Method 3, before we inoculated overnight cultures, the clones were allowed to grow during the day until all of them were in stationary phase, which allows much more efficient and uniform amplification results. This procedure provides a more uniform inoculum for each clone, allowing the overnight cultures to achieve more consistent growth and, consequently, more consistent amplification.

Our analysis indicates that clone growth before PCR is essential for consistent, high-quality amplification from culture. However, growth for periods longer than 16–18 h may have adverse effects on success, leading to a decrease

in product yield. Nevertheless, the most uniform results are achieved if the clones are first allowed a period of 7–9 h for “recovery growth” before the inoculation of overnight cultures. We have discovered that the use of these optimized growth conditions allow for reliable and reproducible amplification of cDNA clones directly from bacterial culture with no need for plasmid template preparation.

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Address correspondence to Dr. John Quackenbush, The Institute for Genomic Research, 9712 Medical Center Dr., Rockville, MD 20850, USA. e-mail: johnq@tigr.org

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**R. Gaspard, S. Dharap,
J. Malek, R. Qi, and
J. Quackenbush**
*The Institute for
Genomic Research
Rockville, MD, USA*