AmpArray™

User's Manual 1.0

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AmpArray™ Product Packing List

Items included:

1. Sealed 96-well PCR plates arrayed with primers (two).
2. Thermostable sealing tapes (two).
3. Oligo dT.
4. Control primer mixture (G3PDH primers).

Items required but not included:

1. 96-well PCR machine with a heated lid.
2. cDNA synthesis materials (Reverse transcriptase, dNTPs mix).
3. PCR reagents.
4. A 96-well agarose gel electrophoresis apparatus. (Such a gel box and combs can be purchased from DAN-KAR CORP, **be sure to ask for multi-channel pipette compatible combs** (13 wells). Stock # 156-EC-H, Tel. 800-942-5542).

Introduction

Recently, gene expression profiling has become an important approach in identifying genes which play key roles in biological pathways and disease pathways. DNA arrays for expression profiling are generally classified into two categories: low density and high density arrays. Both are widely used for high-throughput gene expression profiling for basic research and drug discovery.

Although DNA arrays for gene expression profiling have achieved significant success, they do have some limitations due to the nature of the procedures. The common procedure for all DNA arrays is based on hybridization; therefore, sometimes it is difficult to detect the low abundant transcripts of certain genes that are critical in physiological and pathological functions. Also, the signals of some transcripts can be masked by other non-specific sequences. To overcome the limitation, GeneCopoeia has developed a new expression profiling system, the AmpArray™, to provide researchers an alternative solution. It takes advantages of the high specificity and sensitivity of PCR. It is designed to detect the expressions of the genes in a biological pathway, a disease pathway or a gene family. Each AmpArray ™ is a 96-well PCR plate arrayed with duplicated 48 pairs of primers. Among the primers, one pair primers are specific to the housekeeping gene, G3PDH, and works as a control. The rest of primer pairs are specific to the individual genes grouped with biological relevance.

PCR has been proven to be a simple and reliable method to detect or compare the expression levels of a given gene in different samples; however, a successful amplification of fragments from multiple genes in a 96-well PCR requires that all primer pairs are suitable for one PCR condition. To that end, the primers are meticulously designed by using a highly reliable primer designing algorithm which provides an uniform annealing temperature and similar PCR product sizes so that one set of PCR condition works for all primer pairs.

Application of AmpArray™

1. Focus on gene profiling with selected groups of the genes
2. Detect expression change of the genes with low copy number
3. Be able to detect and discover alternative splicing forms of gene expression
4. Validate data from high density gene chips
5. Detect the effects of RNAi on expression of the related genes

Advantages of using AmpArray™:

1. Easy-to-use and timesaving: no radioactive labeling, no hybridization, obtain data in one or two days.
2. Affordable: no specific equipment required, only a 96-well regular PCR.
3. High-sensitivity: the number of PCR cycles can be adjusted based on the levels of transcripts.

4. High-specificity: each pair of primers is well-selected, and results are confirmable with predicted sizes.
5. Total RNA can be used: primer pairs are selected to minimize the effects of genomic contamination.
6. Discovery of specific alternatively spliced forms in different samples.

We have developed a series of AmpArray™ specific to different biological pathways for you to choose. You are also welcome to provide suggestions on how a particular AmpArray™ should be organized or customized for your interest.

First Strand cDNA Synthesis:

The purpose of this experiment is to generate the first strand cDNAs to be used as a PCR template in an AmpArray™. Total RNAs or PolyA+ RNAs can be used as starting materials for the first strand cDNA synthesis. In general, polyA+ RNA has less genomic DNA contamination and it is recommended for the purpose; however, the materials to be used sometimes are limited and/or some targeted transcripts are very rare that may loss after polyA selection. In these cases, total RNAs shall be used. Several approaches are taken to alleviate genomic DNA contamination that may be encountered during PCR. First, the primer pairs in the AmpArray™ panels are designed to minimize the effects of genomic DNA contamination whenever it is possible; second, an optimal amount of template added to the PCR reaction can also limit the effect of genomic DNA contamination. Users can choose total RNAs or polyA RNAs based on their needs.

Usually, RNA samples are paired for analysis, such as treated versus untreated cells or normal versus abnormal tissues. It is suggested to synthesize the cDNA pair simultaneously. In the following example, a pair of total RNA samples from human THP-1 (monocytic leukemia) with and without the treatment of PMA was used for the cDNA synthesis.

Table 1. Recommended first strand cDNA synthesis volume:

Total RNA (μg)	Poly A RNA (μg)	Total Reaction Volume (μl)
0.5-0.9	0.1-0.9	25
1-5	1.0	50
6-15	2.0-5.0	100

Protocol: an example cDNA synthesis for 10 μg total RNA:

1. Add following to a 1.5ml Eppendorf tube:

Total RNA (1.0 μg/μl)	10 μl
Oligo dT (0.5 μg/μl provided)	2 μl
H_2O	13 μl
	25 μl

2. Heat the mixture to 70ºC for 5 minutes and then put it on ice. Add following to the tube:

5 x MMLV RT reaction buffer (Promega)	20 μl
5 x dNTP mix (2.5 mM each)	20 μl
H_2O	32.5 μl
200 u/μl MMLV RT (Promega)	2.5 μl
	100 μl

3. Mix the tube and incubate it at 42°C for 50 min and then shift it to 70°C for 10 minutes.

4. Store the cDNAs at –20°C.

Semi-Quantitation of Synthesized cDNAs:

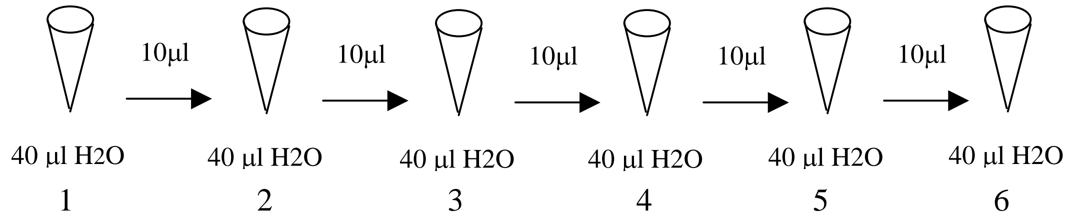
The purpose of this experiment is to semi-quantitate the amount of the cDNAs in comparing groups. PCRs are performed by mixing G3PDH primers with a serial of dilutions of the newly synthesized cDNAs. The result sets the amount of cDNAs to be used in one panel of AmpArray™. **The amount of cDNAs to be added should be sufficient to amplify rare transcripts, and two cDNAs should contain equivalent G3PDH transcripts, namely, it is "normalized loading of templates".** Other methods such as real-time PCR can also be used for the purpose.

Protocol

1. Make a series of six dilutions of the cDNAs as illustrated in the diagram. The dilution between a concentration and the next one is five folds. The dilution factors for the concentrations are 1/5 , 1/25, 1/125, 1/625, 1/3125 and 1/15625 respectively:

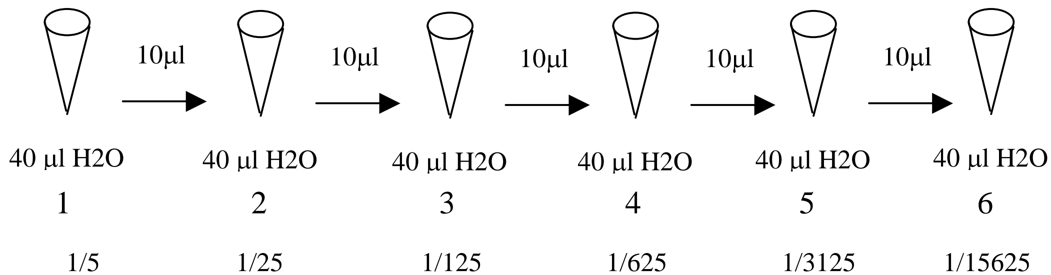
cDNA #1 (untreated)

10 µl



cDNA #2 (treated)

10 µl



2. Take a 12-tube strip and transfer 2µl from each diluted cDNA to a correspondent tube in the strip.

3. Prepare a PCR pre-mix as following:

10 x PCR buffer	28 µl
10 x dNTP (2 mM each)	28 µl
G3PDH primer mix (5 pmole/µl each, provided)	28 µl
H_2O	154 µl
Taq polymerase (1u/µl)	14 µl
	252 µl

4. Aliquots 18µl of the pre-mix to each tube in the strip starting from the most diluted tube to avoid contamination.

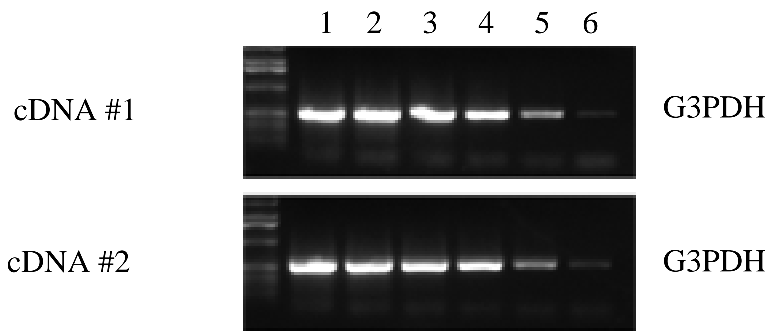
5. Run PCR with the condition as:
   94 °C (3 min)

   30 cycles as following:
   94 °C (30 sec)
   62 °C (30sec)
   72 °C (1.5 min)

   Extension 72 °C 5min

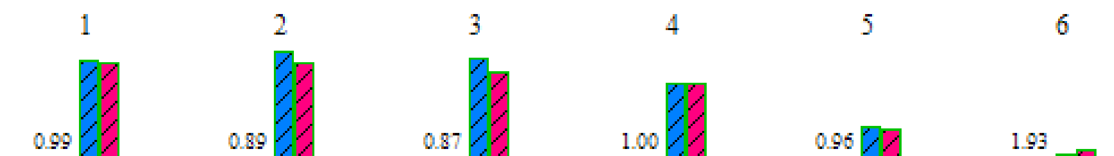
6. After the PCR, add 4μl of 6X loading buffer to each tube and load 10μl of the sample to a 1% agarose gel electrophoresis to check the PCR results. A sample data is shown in Fig. 1.

Fig. 1. Comparison of amplified G3PDH products between the cDNA templates from cells without the treatment of PMA (cDNA #1) and with the treatment of PMA (cDNA #2).



7. Quantitate the product with a gel analyzing software. Although the amount of PCR products can be visually determined, it is recommended to use a gel analyzing software. Many gel documentation systems with standard software for quantifying DNA bands resolved in agarose gel can be used for the purpose. The following figure illustrates the results generated by GelPicAnalyzer 1.2 from GeneHarbor Inc. The software is specially designed to analyze products from a 96-well PCR.

Fig. 2. Data acquired from the gel image by using GelPicAnalyzer 1.2 were shown in Fig. 1.. The blue bars represent data from the bands of cDNA #1 and red bars represent data from cDNA #2. The numbers at the lower left corner of each bar pair indicate the ratio of readings from cDNA #2 divided by that from cDNA #1.



8. **Determine the normalization factor**. The unsaturated band should be selected from each cDNA sample for comparison. For example, as shown above, lane 5 for untreated and treated samples contains ideal bands for comparison. The factor is 0.96. Pair #5 is the best because the PCR is not saturated and the amount of products is well above the background. Although pair #6 is not saturated, it has little product and easy to be affected by background reading. It is important to point out that this is a semiquantitive method and therefore may not be accurate as real-time PCR; nevertheless, it suffices the purpose to control the amount of templates between two samples.

9. **Select an optimal concentration of cDNAs for AmpArray™**. The concentration of cDNA is important to get meaningful results. In general, it is recommended to use the cDNA dilution that is two hundred fold higher than that of the cDNAs resulting in semi-saturated amplification of G3PDH (such as #5 dilution in Fig. 1). For example, as shown above, the optimal dilution factor should be [(1/3125) X 200], which is namely about 15 fold dilution of the original cDNA mixture. Two micro liters (2 μl) of diluted cDNA are added to each tube in the AmpArray™. Thus, the total amount of synthesized cDNA can be used for more than ten panels.

<u>AmpArray™ PCR</u>:

1. Take out an AmpArray™ plate from –20 °C and put it on ice.
2. Mark two 1.5ml tubes with correspondent names of cDNAs, i.e. control or treated.
3. Prepare two premixes on ice as following:

	Tube 1 (cDNA 1) (μl)	Tube 2 (cDNA2) (μl)
10x PCR buffer	110	110
10x dNTP (2mM each)	110	110
Template (diluted)	110	110
Taq polymeras	55units/55μl	55units/55μl
H_2O	715	715
Total	1100	1100

4. Remove the sealing tape from each plate and dispense 20 μl of cDNA1 to each well in the odd number column (1, 3, 5, 7, 9, and 11). This can be achieved by using a multi-channel pipettor: first divide the 1000 μl premix into 8 tubes and then transfer 20μl of the premix to each tube.

5. Aliquots 20 μl of cDNA2 to each well in the even number column (2, 4, 6, 8, 10, and 12).

   Note: The premix may not be enough due to the system error of a pipettor, and that could be very annoying. To avoid the problem, please try to make extra premix.

6. Seal the plate with a new thermostable PCR plate sealing tape (provided) and mount the plate to a 96-well PCR machine. Run PCR using the following condition:

   94 °C (3 min)

   32 cycles as following:
   94 °C (30 sec)
   66 °C (30sec)
   72 °C (1.0 min)

   Extension 72 °C (5min)

   4 °C for sample preservation.

7. During the PCR, prepare a 1.2% agarose gel with enough wells for the 96 PCR products and DNA markers. (Such a gel box and combs can be purchased from DAN-KAR CORP, Stock # 156-EC-H, Tel. 800-942-5542)

8. Add 10 μl 3×DNA loading buffer to each tube. After mixing well, load 13 μl of each sample to a well of agarose gel. Take caution to avoid sample contamination. Run a 1.2% Agarose gel at 3v/cm. Take a picture with a digital UV light-camera and save the picture file as a Tiff file (.tif).

9. Up or down regulated genes and alternatively spliced form of genes can be visualized and quantified by using a gel image analyzing software.