RAPID-SCAN™

GENE EXPRESSION PANELS

Applications Guide

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A. Package Contents and Storage Conditions

Components included:

- Two identical, sealed, 96-well plates containing twenty-four dried, serially-diluted, PCR-ready, first-strand cDNAs.
- Two adhesive cover sheets for sealing the 96-well plates prior to performing the PCR reaction.
- Control primer pair for detection of β-actin cDNA (1nmol each/200 ul for 100 reactions).
- Free sample of DNA Quanti-Ladder™, a ready-to-use molecular marker for easy quantitation and fragment-size determination (100 ul for 20 lanes).
- Free sample of Rapid-Load™, a direct loading dye for electrophoresis of PCR products (1.5 ml for 200 reactions).

The above components are shipped at room temperature, but should be kept at 4°C for long-term storage. If properly stored, they have a 12-month shelf-life.

Components not provided:

- Reagent mix for PCR* amplification (PCR buffer, Taq DNA polymerase, dNTPs, gene-specific primers).

*The PCR technology is covered by patents issued to Cetus Corporation, and is owned and licensed by Hoffmann LaRoche Molecular Systems, Inc. Purchase of any of OriGene's PCR-related products does not convey a license to use the PCR process, covered by these patents. Purchasers of these products must obtain a license to use the PCR process before performing PCR.

B. Introduction

With the availability of technologies to support high-throughput gene discovery, comes the need for complementary technologies for large-scale functional discovery. An essential first step in this process is to determine the comprehensive expression profiles of the newly-identified genes, information which may provide key clues to our understanding of biological functions. OriGene Technologies, Inc. is a biotechnology company focused on developing products to support high-throughput gene cloning and analysis. We have now developed the Rapid-Scan™ Gene Expression Panel to serve this market.

Rapid-Scan™ is a PCR-based system, using high-quality, first-strand cDNAs derived from 24 different tissues and/or developmental stages, to generate a 'comprehensive' expression profile of any cloned gene or identified expressed sequence tag (EST) from either human or mouse. Individual first-strand cDNAs have been tested to assure that low abundance and long transcripts are represented, and have been normalized using β-actin cDNA as internal standard. The 24 cDNAs were then serially-diluted over a 4-log range and arrayed onto a 96-well PCR plate, in order to assure that the amplification

reaction will be within the linear range and, hence, to facilitate semi-quantitative determination of relative mRNA accumulation in the various tissues and developmental stages. With this non-radioactive technique, one can generate a comprehensive expression profile for any given human or mouse gene in just three hours. All that is required is a 96-well PCR using your gene-specific primers, followed by electrophoretic analysis of the products on an agarose gel. Rapid-Scan™ is designed to be a user-friendly product.

Benefits of using the Rapid-Scan™ panel:

- Fast and non-radioactive (no radiolabeling of probe / no blot hybridization / no filter washing).
- Simultaneous examination of 24 different tissues and/or developmental stages.
- High-sensitivity and semi-quantitative.
- Simultaneous analysis of alternatively-spliced forms or members of a gene family.

C. Production and quality assurance:

We assembled the Rapid-Scan™ panels by selecting either 24 frequently-studied human tissues or 24 major mouse tissues and developmental stages (see Figures 1 and 2). To avoid detection of individual differences in gene expression, we have whenever possible pooled tissues from multiple individuals. For the human panel, tissues were from individuals of different ethnicity. For the mouse panel, adult tissues were from outbred Swiss Webster and breast tissues from outbred CD1 mice.

Total RNA was isolated and subjected to oligo(dT) selection. The recovered poly(A^+) RNA was then examined by Northern blot hybridization, using a β-actin cDNA probe as control, to confirm RNA intactness. Poly(A^+) RNA with a high percentage of intact β-actin transcripts was used to synthesize first-strand cDNA, using oligo(dT) primers and MMLV reverse transcriptase. Individual cDNA pools were confirmed to be free of genomic DNA contamination and to contain complete reverse transcripts of selected rare and long mRNAs, such as those for the transferrin receptor (5 kb) and the ataxia telangiectasia gene (9.4 kb). The amount of first-strand cDNAs from each tissue was then adjusted to contain an equivalent concentration of β-actin reverse transcripts (see Figure 3). Each cDNA was diluted in water to a series of four concentrations (labeled 1000X, 100X, 10X and 1X), with the lowest concentration (1X) being approximately 1 pg/ul. The diluted cDNAs were subsequently arrayed (2.5 ul) onto a 96-well PCR plate in the order indicated in Figure 1 (human) and Figure 2 (mouse).

D. Protocol for use of the Rapid-Scan™ panels:

Note before starting experiment:
The key to success in the use of this gene expression panel is the design of a pair of gene-specific and PCR-efficient primers. The following rules for primer design are suggested:

1. The primers should ideally correspond to the coding region of the gene of interest. The positions of the forward and the reverse primers should be between 300 and 1000 bp apart, so as to facilitate efficient template amplification and easy detection of the PCR product in an agarose gel.

2. Each primer should be 18 to 22 bases long and has a G/C content between 50-55%. They should lack any strong secondary structure and must not self-anneal or cross-anneal. The G/C's should be evenly distributed along the oligos and there must not be more than 3 G's in a row.
3. If possible, a pilot PCR amplification should be performed using the gene-specific primers and a test template, such as a plasmid cDNA clone or a first-strand cDNA preparation, that is known to contain the target sequence of interest.

Experimental Procedure:

1. Take the Rapid-Scan™ plate out from the 4^{0}C refrigerator, and allow it to warm at room temperature.
2. Prepare the PCR pre-mix as follows:

Stock Solution	ul	Final Concentration
10X PCR Buffer	250	1X PCR Buffer
10X dNTP (2.5 mM each)	250	1X dNTP (0.25 mM each)
Primer 1 (10 pmol /ul)	100	10 pmol/well
Primer 2 (10 pmol /ul)	100	10 pmol/well
H_2O	1790	
Taq DNA Polymerase (5U/ul)	10	0.5 U/well
Total	2500	

3. Remove the sealing film from the Rapid-Scan™ plate. Add 25 ul of the PCR pre-mix to each well, avoiding cross-contamination during pipetting. This can best be achieved using a multichannel pipettor.
4. If using a thermocycler with a heating lid, go to step 5; otherwise, add 15 ul mineral oil to each well to prevent evaporation.
5. **Cover the top of the plate with a new adhesive cover sheet (provided). Seal each well tightly by pressing the cover around each well. Remove any air bubbles that may have been trapped at the bottom of the tubes by gently tapping the plate.** Let the plate sit on ice for 15 minute to allow the dried cDNA to dissolve. No pipetting or vortexing is needed to resuspend the DNA.
6. **Mount the plate snugly onto a 96-well thermocycler, so that each well has a tight contact with the heating block. Tighten the lid to ensure a direct contact between the lid and the top of the 96-well plate to prevent any air leakage from the wells.**
7. Use a thermocycling parameter optimized for your gene-specific primers. We recommend the following conditions: pre-soak at 94^{0}C for 3 minutes, followed by 35 cycles of denaturation at 94^{0}C for 30 seconds, annealing at 55^{0}C for 1 minute, and extension at 72^{0}C for 2 minutes. Final extension will be at 72^{0}C for an additional 5 minutes.
8. Prepare a 1% agarose gel to accommodate 96 samples, plus molecular weight markers.
9. When the reaction is completed, remove the cover sheet and add to each well 12.5 ul of 2.5X DNA loading buffer which can be prepared by diluting the 5X Rapid-Load Buffer (provided) with 1 vol. of water.
10. Load 15 ul of each sample to the gel, starting from the lowest to the highest concentration and changing pipet tips after each tissue group and arranging the samples in such way that the row with the lowest concentration (1X) is near the positive

electrode. Include molecular weight markers as desired (5 ul Quanti-Ladder™). Store the left samples at -20 °C for other uses mentioned in the following section.

11. Run the agarose gel and take an EtBr picture after its completion.

Two individual 96-well plates are provided for your convenience. Ideally, the two plates may be used for the analysis of two different genes or ESTs. Alternatively, after obtaining an adequate expression profile for a test gene, the second plate may be used for determining the β-actin profile using the control primers provided. In the event that the gene-specific primers gave only a marginal signal with the first plate, indicative of the detection of a very low abundance transcript, the second plate may be used with an increase in amplification cycle and/or a modification in annealing temperature.

If both 96-well plates have been used for gene-specific primer analyses, one may still consider performing a β-actin control with the used plates. Specifically, we suggest you taking one-hundredth of the 1000X PCR reaction product to perform a second PCR reaction with the β-actin primers provided. In this case, the loading buffer used to stop the first PCR reaction must not contain either EDTA or SDS. Should the product of the first PCR reaction using the gene-specific primers overlap in size with the expected β-actin product (640 bp for human and 570 bp for mouse) derived from the primer pair provided, one may have to synthesize new primers such that the anticipated product size will be distinct from that of the gene-specific product, thus avoiding ambiguity in data interpretation. It has to be cautioned, however, that the results obtained with a used plate cannot be as quantitative and reproducible as one may expect, and that the β-actin control (if deemed necessary) is best done on a fresh 96-well plate.

E. Sample Gene Profiling by Rapid-Scan™

To facilitate comparison of transcript accumulation in different tissues, the first-strand cDNAs have been normalized using β-actin as internal control. Figure 3 shows equivalent β-actin distribution in all 24 human tissues, when products of a 30-cycle PCR amplification (using primers provided with the Rapid-Scan™ panels) were analyzed on a 1% agarose gel. Of the 4 cDNA concentrations used (labeled from 1000X to 1X), only the lowest concentration (1X) gave an amplification signal that was in the linear range. The 10X concentration signal was just subplateau and the signals for both 100X and 1000X were already at plateau. It should be noted that the slight variation in β-actin transcript accumulation was detected only at cDNA concentrations which were in the linear range but became obscured when the cDNA concentrations were at saturation. This finding illustrates the need to perform the PCR analysis over a range of cDNA concentrations. The concentration range selected assures a better chance of detecting any unknown gene transcript, which almost invariably will accumulated at a lower abundance level than the β-actin transcript.

As an example to illustrate the use of the Rapid-Scan™ panel to determine the relative accumulation of the muscle-specific a-actinin 2 transcript, a primer-pair derived from the target gene of interest was used (Figure 3). As expected, a-actinin 2 mRNA was detected in abundance only in skeletal muscle and heart muscle. There was less than a 10-fold difference in mRNA accumulation between the two tissues; at 10X cDNA concentration, skeletal muscle was about 2.5-fold higher than cardiac muscle. The a-actinin 2 transcript was not detected in any other tissues other than the brain, where the a-actinin 2

protein has been shown to anchor the N-methyl-D-asparate (NMDA) receptor on neurons. From a comparison of PCR signals at different cDNA concentrations, it was observed that the a-actinin 2 mRNA level in the brain was one-hundredth that in skeletal muscle.

The Rapid-Scan™ panel may also be used to quantitate the relative usage of alternate splice sites in different tissues or differentiation states. As an example, we have compared the expression of two classes of transcripts derived from the amyloid precursor protein (APP) gene using a single primer pair (Figure 3). The APP695 transcript differs from the APP770 transcript by a 225-bp (75 amino acid) deletion, resulting from alternate RNA splicing. While APP770 was detected in all adult tissues, it was not present in the two fetal tissues. On the other hand, APP695 was most abundant in the brain, regardless of developmental stage. It was, however, not absolutely specific to this tissue but was also found in lower abundance in testis and in trace amounts in other sites.

While there continues to be a debate as to whether tissue-specific genes are a rarity rather than a commonality in the human body, the Rapid-Scan™ panel can find utility in defining the level of 'escape' expression of suspected tissue-specific genes in unsuspecting tissues. Such findings may have implications both in the development of diagnostics and the identification of therapeutic targets. For example, analysis of the prostate-specific NKX3.1 gene, a homeodomain-containing transcriptional factor, revealed expression in testis, salivary gland, and PBL, at levels almost two logs below that in adult prostate (Figure 3).

The Rapid-Scan™ panel is also an effect tool to obtain an expression profile of an uncharacterized EST. The result shown in figure 4 demonstrated an example for such use. Using a mouse Rapid-Scan panel, a mouse EST sequence is found expressed in skin, stomach and 19 day old embryo.

In the use of the Rapid-Scan™ panels to determine the relative quantitation of mRNA accumulation, one has to be reminded that the PCR amplification has to be carried out with excess primers and that the PCR readout has to be within the linear range. This frequently implies that one should be cautious to not overamplify the PCR product by incorporating too many cycles of amplification. However, too few cycles of amplification may not suffice to detect transcripts with a low abundance of accumulation. Since it is difficult to predict a balance between sensitivity and nonlinearity, we suggest that investigators start by performing a 35-cycle amplification with the first 96-well plate to assure detection of a PCR signal and, if necessary, use the second 96-well plate for optimization and quantitation.

F. Trouble-shooting Guide

1. No PCR product detected

- May have omitted a PCR component: Be sure to use a written checklist when assembling the reaction mix.
- May have used an inappropriate annealing temperature: PCR condition was suboptimal. If at all possible, test your primers against a known template, which can be diluted cloned DNA, to determine the optimal condition for amplification.

- May be due to poor primer design: Proper choice of primers is important for a successful PCR amplification. While difficult to design a perfect primer pair, those with strong secondary structures and those that can self-anneal/cross-anneal should be avoided.

2. More than one PCR product detected

- Existence of multiple distinct products: It is possible that all of the products are specific, resulting from the amplification of either alternatively-spliced mRNAs or transcripts derived from distinct members of a gene family. This may be resolved by either DNA sequencing of the PCR products or use of nested primers for reamplification.
- Detection of both specific band(s) and a background smear: This is likely the result of the low annealing temperature used, which gave rise to both specific component(s) and non-specific background noise. The non-specific components may be reduced by an increase in the stringency of the PCR amplification.

NOTE: FOR RESEARCH PURPOSES ONLY! NOT FOR DIAGNOSTIC OR THERAPEUTIC USAGE.

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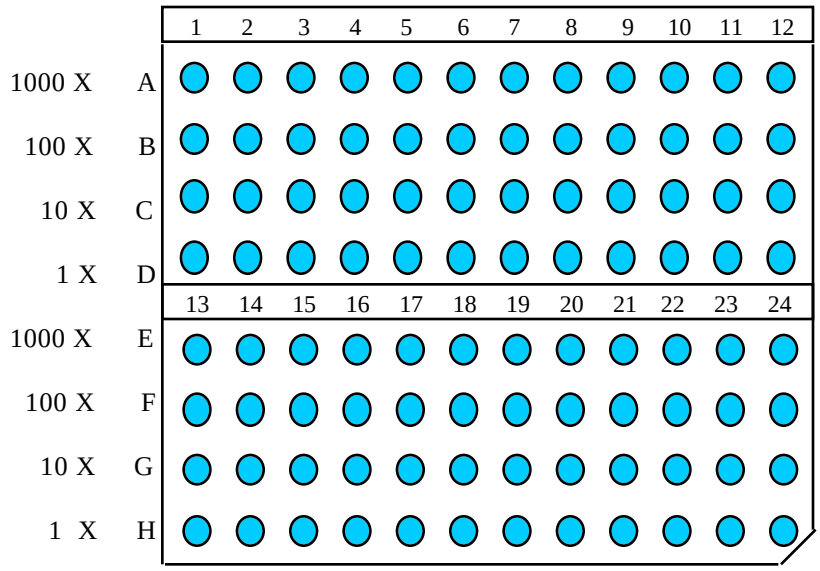
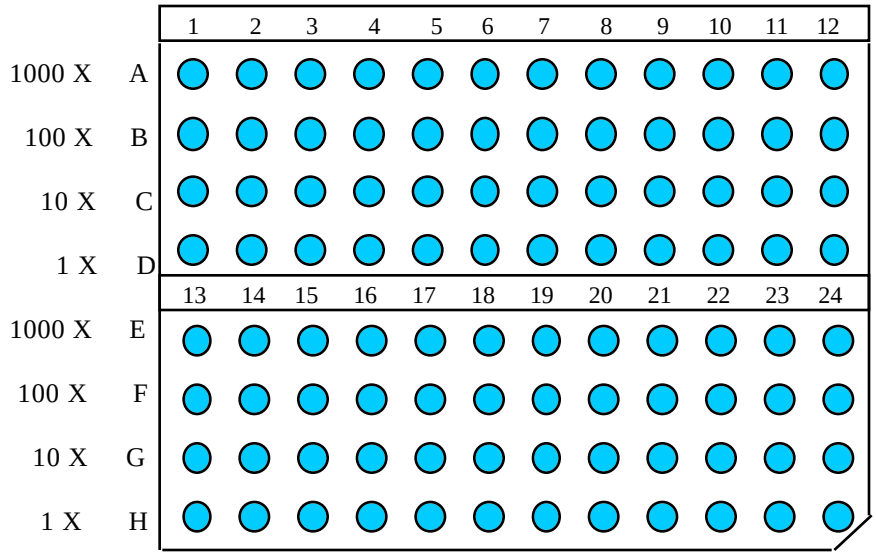
Figure 1. Human Rapid-Scan™ Panel



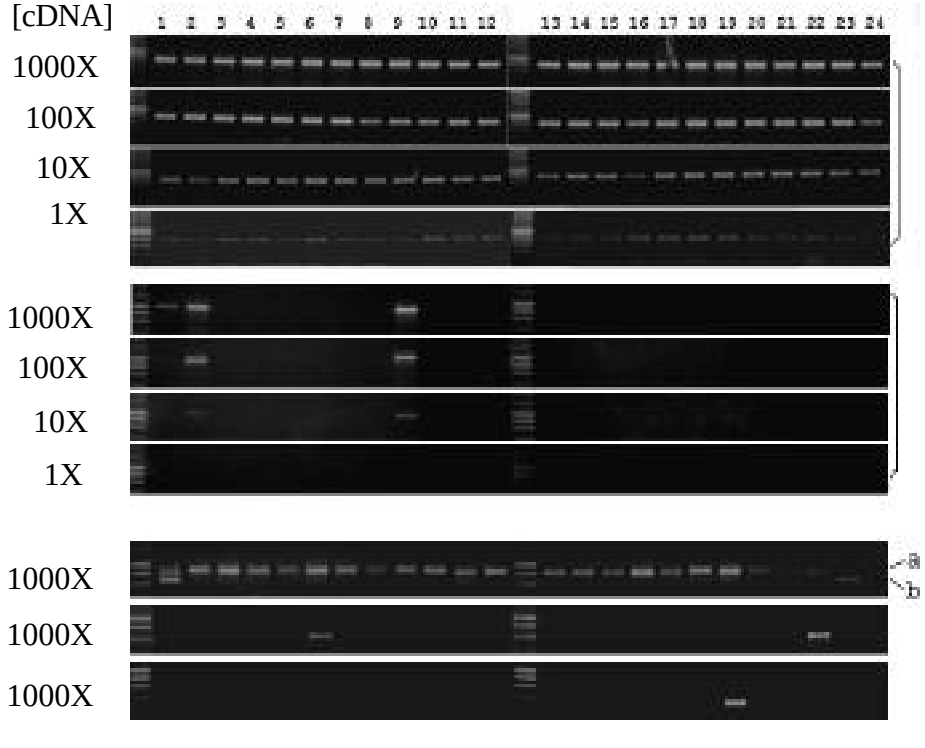
1. Brain 2. Heart 3. Kidney 4. Spleen 5. Liver
6. Colon 7. Lung 8. Small Intestine 9. Muscle 10. Stomach
11. Testis 12. Placenta 13. Salivary Gland 14. Thyroid Gland 15. Adrenal Gland
16. Pancreas 17. Ovary 18. Uterus 19. Prostate 20. Skin
21. PBL 22. Bone Marrow 23. Fetal Brain 24. Fetal Liver

Figure 2. Mouse Rapid-Scan™ Panel



1. Brain 2. Heart 3. Kidney 4. Spleen 5. Thymus
6. Liver 7. Stomach 8. Small Intestine 9. Muscle 10. Lung
11. Testis 12. Skin 13. Adrenal Gland 14. Ovary 15. Uterus
16. Prostate Gland 17. Embryo e8.5 18. Embryo e9.5 19. Embryo e12.5 20. Embryo e19
21. Breast/Virgin 22. Breast/Pregnant 23. Breast/Lactating 24. Breast/Involuting

Figure 3. Sample Profiling of Genes by Use of the Human Rapid-Scan™ Panel



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4. Brady HJ, Lowe N, Sowden JC, Edwards M, Butterworth PH. (1991) The human carbonic anhydrase I gene has two promoters with different tissue specificities. Biochem. J. 277: 903-5.

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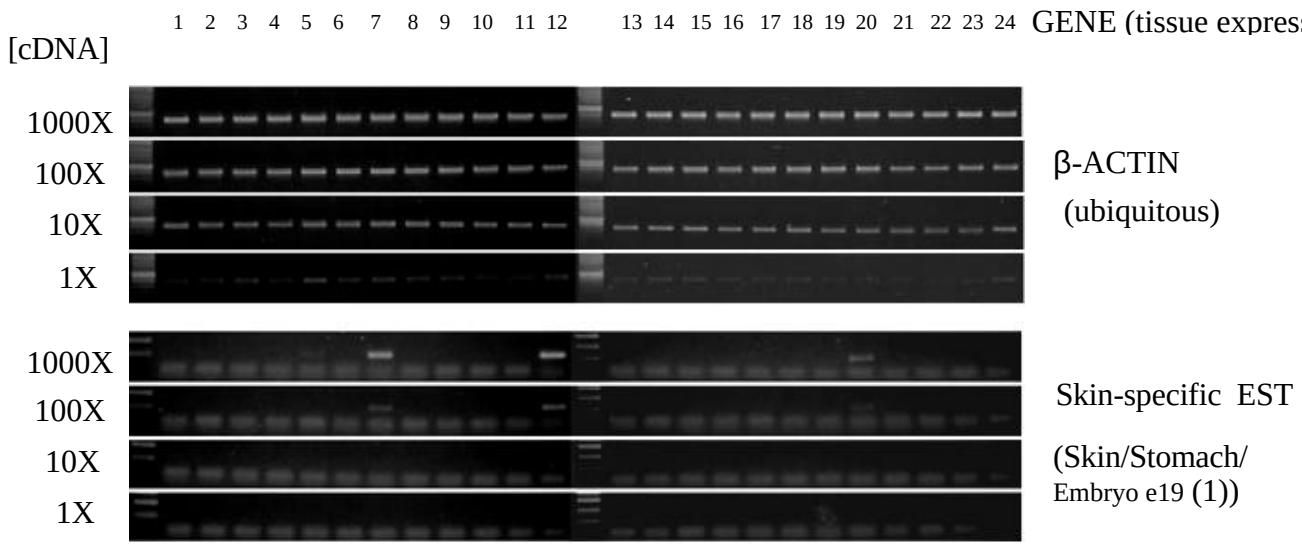
Figure 4. Sample Profiling of Genes by Use of the Mouse Rapid-Scan™ Panel



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21. Breast/Virgin	22. Breast/Pregnant	23. Breast/Lactating	24. Breast/Involuting	

1. A mouse EST clone (gb: AI391093), similar to a human skin-specific cDNA (xp32). Zhao XP, Elder JT (1997) Positional cloning of novel skin-specific genes from the human epidermal differentiation complex. Genomics 45: 250-8.