

# Sure-Race™

## Multi-Tissue RACE Panels

### APPLICATION GUIDE

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# PACKAGE CONTENTS AND STORAGE CONDITIONS

- Four identical, sealed, 48-well plates containing two dilutions of 24 human or 24 mouse RACE-ready cDNAs
- Adapter primer 1 (ADP1, 10  $\mu$ M), outer primer for first-round PCR
- Adapter primer 2 (ADP2, 10  $\mu$ M), inner primer for second-round (nested) PCR
- Transferrin receptor primer 1 (TRP1, 10  $\mu$ M), positive-control primer for first-round PCR
- Transferrin receptor primer 2 (TRP2, 10  $\mu$ M), positive-control primer for second-round (nested) PCR
- Free sample of DNA Quanti-Ladder™, a ready-to-use molecular marker for easy quantitation and fragment-size determination

*The above components are shipped at room temperature, but should be kept at -20°C upon arrival.*

- Sure-Race™ Applications Guide

## Other required Reagents

- PCR reagents (Taq DNA polymerase, 10 mM dNTPs, PCR buffer, sterilized dH<sub>2</sub>O)
- GSP1 gene-specific, outer primer for first-round PCR (T<sub>m</sub> = 68-70°C)
- GSP2 gene-specific, inner primer for second-round (nested) PCR (T<sub>m</sub> = 65-68°C)

## Related Products

TrueClone™ cDNA clones <http://www.origene.com/cdna/>

HuSH™ shRNA Plasmids <http://www.origene.com/rna/>

Validated Antibodies <http://www.origene.com/antibody/>

First Strand cDNAs [http://www.origene.com/geneexpression/strand\\_cdna/](http://www.origene.com/geneexpression/strand_cdna/)

Northern Blots [http://www.origene.com/geneexpression/rna\\_blots/](http://www.origene.com/geneexpression/rna_blots/)

# INTRODUCTION

## Overview

Traditional cDNA libraries frequently yield clones which are incomplete and where sequences representing the mRNA 5' ends are missing. The RACE technology, a PCR-based approach for "rapid amplification of cDNA ends", has been particularly useful for completing the missing portions of cDNA clones and yielding full-length sequences of expressed regions of genes.

To expand the utilities of the RACE technology, OriGene has now developed a PCR-ready RACE panel which will allow simultaneous 5' end analysis of transcripts from 24 individual human tissues or 24 mouse tissues and developmental stages. Sure-RACE™ panels, consist of double-stranded cDNA arrayed multi-well plate. Sure-RACE™ is designed with the recognition that an increasing number of genes

have been identified that utilizes alternate RNA start-sites resulting from the use of tissue-specific or developmental stage-specific transcriptional promoters. As well, an increasing number of genes have also been found to utilize alternate 5' exons, sometimes resulting in protein products with different N-terminal amino acid sequences.

By simply performing a nested PCR analysis, using two contiguous adapter-specific primers provided with the kit and two contiguous gene-specific primers designed by the investigator, the Sure-RACE™ panels will markedly increase the probability of completing the 5' ends of transcripts which are rare or have complex secondary structures, by surveying many more tissues where each may have yielded a different proportion of full-length reverse transcripts because of differences in starting RNA intactness, relative RNA accumulation, and/or efficiency of cDNA synthesis, allow the identification of alternate RNA start-sites within the same tissue, in different tissues or at different developmental stages, findings which may lead to the suggestion of the use of alternate transcriptional promoters, and provide evidence for alternate RNA splicing at the 5' end, such as the use of alternate first exons or an identical first exon but alternate downstream exons, which may result in proteins with nonidentical amino acid sequences. Any or all of these information are derived from direct sequencing of the PCR products generated by the human or mouse Sure-RACE panels.

Special care and important modifications have been incorporated into the development of the Sure-RACE™ panels, in order to assure that the cDNAs used for analysis are of the highest quality. They include assurance that the starting RNAs are intact, use of an elevated temperature and a thermostable enzyme to allow read-through of RNA conformations during first-strand cDNA synthesis, and use of a technology which avoids loss of 5' end sequences during second-strand cDNA synthesis.

## **PRODUCTION AND QUALITY ASSURANCE**

Development of Sure-RACE™ Multi-Tissue RACE Panels began with the selection of high-quality mRNAs. The integrity of each of the polyA<sup>+</sup> RNAs was determined by Northern blot analysis using beta-actin cDNA as the hybridization probe. Only mRNAs that fulfill stringent criteria of intactness were used for cDNA synthesis.

It is well-established that mRNAs have complex secondary structures which may act as strong-stops during first-strand cDNA synthesis using standard reverse transcriptase (RT). As a consequence, the resulting single-stranded cDNAs may not be representative of the starting mRNAs in that their 5' ends may not be reverse transcribed. OriGene has successfully used a dual-cycling procedure to overcome this problem. Specifically, the initial cDNA synthesis was performed using standard procedures with MMLV RT at 42°C, followed by a second round of synthesis with thermostable Tth DNA polymerase at 75°C. Cycling of high-processivity / low-thermostability enzyme and low-processivity / high-thermostability enzyme, which will facilitate read-through of RNA secondary structures, was repeated a second time to insure the

efficiency of the reaction.

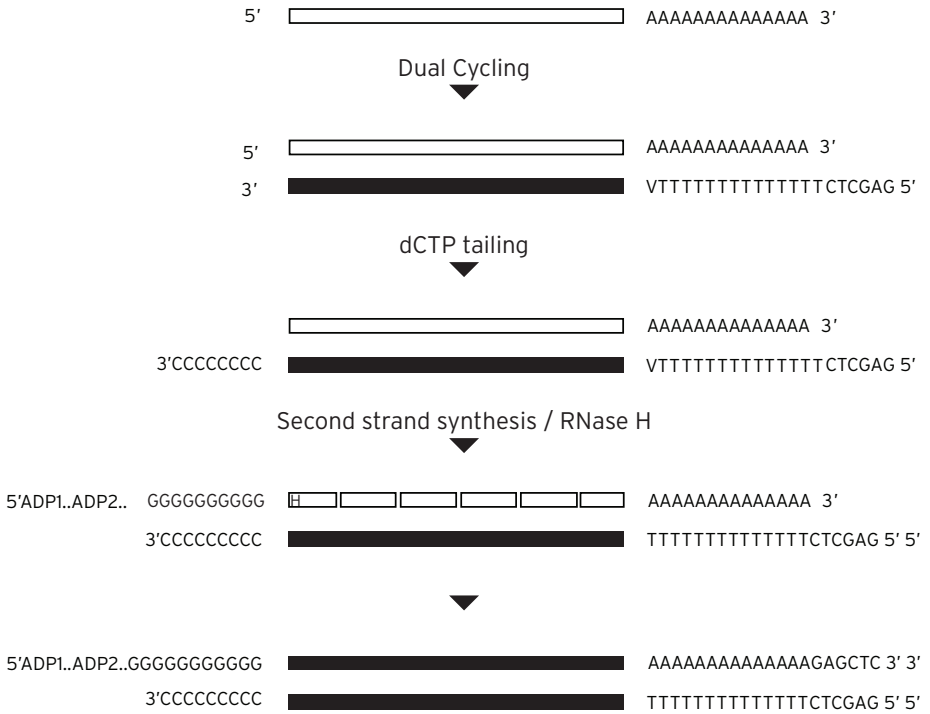
The quality of the first-strand cDNA products was monitored by RT-PCR analysis of two long transcripts, that of the ataxia telangiectasia (ATM) gene which is ~12 kb in length and of the DNA-dependent protein kinase (DNA-PK) gene which is ~13.5 kb long. The efficiency of first-strand synthesis was also improved by using a one-base anchor (A, C, or G residue) at the 3' end of the oligo(dT) primer.

Conventional double-stranded cDNA synthesis involves the blunting of the cDNA, which results in the loss of 5' end sequences of 20-200 nucleotides. To avoid sequence loss, the first-strand cDNAs were tailed at their 3' ends with dCTP (see Figure 1). The terminal transferase reaction was optimized to produce a tail of 10-15 dC residues. Second-strand cDNA synthesis starts with the annealing of an adapter-oligo(dG) primer to the oligo(dC) tail at the 3' ends of the first-strand cDNA molecules. *E. coli* polymerase I was used for second-strand synthesis to reduce the nucleotide-incorporation-error rate. Additionally, RNase H was added to nick the mRNA and to allow internal priming. Finally, T4 DNA ligase was added to facilitate joining of fragments primed by oligo(dG) and nicked mRNA.

The Sure-RACE™ panels were subjected to stringent quality control analysis. More than 12 genes for both the human and mouse panels, have been successfully tested –they include some very long or rare transcripts, as well as some with alternate transcriptional start-sites, and some with differential 5' splicing. Data from these examples are found at the end of this manual. Sure-RACE panels are ideal for completing the 5' end sequence of your cDNAs or to characterize the 5' ends of tissue-specific and alternately-initiated or differentially-spliced mRNAs. Sure-RACE may also be used to clone full-length cDNAs of previously-identified expressed sequence tags (ESTs).

Sure-RACE™ panels were assembled carefully from 24 important human tissues or 24 major mouse tissues and different developmental stages. For the human panel, tissues were from individuals of mixed ethnicity. For the mouse panel, adult tissues were from outbred Swiss Webster mice and breast tissues from outbred CD1 mice. The RACE-ready cDNAs are provided in two concentrations (5X and 1X), to accommodate the analysis of both rare and abundant transcripts. They are arrayed into a 48-well (6 x 8 wells) PCR panel in the order indicated in Figure 2 (human) and Figure 3 (mouse). Four panels are provided per order, to allow replication of the findings and assurance of the specificity of the observed RACE products.

**Figure 1. General procedure for production of Sure-RACE™ cDNAs.**



Sequences of the two adapter primers are: ADP1 5' CGGAATTCGTCAGCTCAGCG 3' and ADP2 5' AGCGCGTGAATCAGATCG 3'. H = A, C or T. V = A, G or C.

# METHODS

## Protocol for Use of Sure-Race™ Panels

RACE is performed using two rounds of PCR. The first round uses the ADP1 and GSP1 outer primers to enrich for the specific cDNAs, and the second round uses the ADP2 and GSP2 inner primers to further amplify the specific target(s). 20 PCR cycles are routinely performed in the first round, followed by 30-35 cycles in the second round (nested) PCR. The specificity of the individual GSP is a critical factor. Because one GSP may be more specific than the other, sometimes 30 cycles in the first round may be optimal, followed by 20-25 cycles in the second round.

After first-round PCR, the reaction product is diluted 1:10 with dH<sub>2</sub>O. The diluted samples are used for the second-round (nested) PCR. It is recommended that more stringent conditions be used for the first-round amplification. Once the desired cDNA has been enriched in the first round, the PCR conditions for the nested PCR can always be optimized. The cycle number of the second round should be 30 cycles for a relatively abundant transcript and 35-40 cycles for a low abundance (rare) transcript.

Hot-start PCR may improve the specificity and yield of desired PCR products, but is not essential for use of our Sure-RACE™ panels. Setting-up the PCR reactions on ice and then transferring the PCR panel onto a pre-heated block of the thermal cycler is usually sufficient for specific amplification. In order to reduce the background and non-specific PCR products, we strongly suggest using our modified touch-down programs for both rounds of PCR amplification. A schematic view of the procedure is described in Fig. 4.

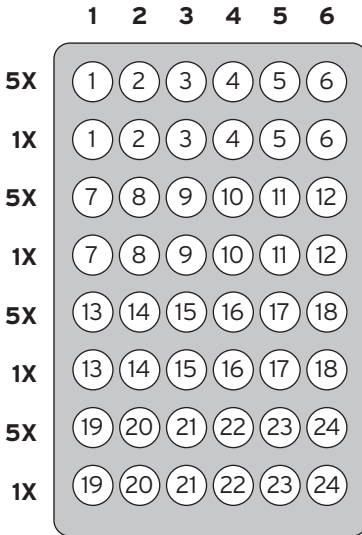
The choice of gene-specific primers is probably the most critical factor for a successful RACE analysis. The GSP1 and GSP2 primers should be compatible with the two adapter primers. The DNA melting temperature (T<sub>m</sub>) of ADP1 and ADP2 are 63°C and 61°C, respectively. In order to use a touch-down PCR program we suggest to design the gene-specific primers with a T<sub>m</sub> around 65~68°C. The T<sub>m</sub> of GSP1 should be around 68°C, and the T<sub>m</sub> of GSP2 should be around 65°C. With a T<sub>m</sub> of 65-68°C, the length of your oligonucleotides should be 22-26, and the G-C content should be 45-55%. It is strongly suggested that primer-design software be used to design the GSP's to avoid primer-dimer formation, secondary structures, and self-complementarity. Too low a T<sub>m</sub> and an incorrect internal stability profile can lead to formation of false-positive bands. Other primer-designing tips include:

- The last nucleotide at the 3' end should preferably be a G or C for tighter binding at the priming site
- All four bases should be evenly distributed and there should not be long stretches of Gs or Cs (more than 3)

- The stability at the 3' end of an oligonucleotide should not be too high; stronger hybridization strength at the 3' portion of the oligonucleotide will cause false priming (8-10 bases are sufficient to prime polymerization)
- The expected length of Sure-RACE PCR products should range from 100-2,000 bp when using regular Taq DNA polymerase; 2,000-5,000 bp when special Taq DNA polymerases are used (e.g. Takara EX-Taq); it is more efficient to amplify smaller fragments. For a fragment above 5,000 bp, long PCR strategy should be used.
- Leave at least 100 bp at the 5' end of your known sequence when you design the second gene-specific primer. The known sequence will enable you to confirm the RACE products by sequencing.

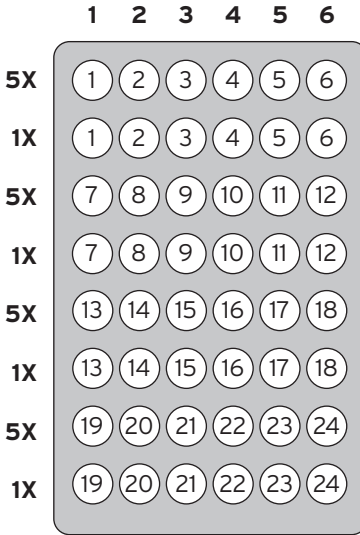
While no software program will guarantee the success of GSP primers in performing RACE, and sometimes it is difficult to choose a satisfactory primer from a limited stretch of EST sequence, adherence to these rules whenever possible will increase the probability of success.

**Figure 2: Sure-Race Human Panels (two 48-well plates)**



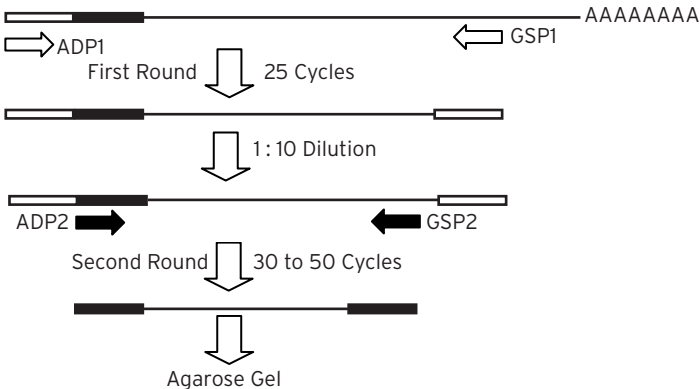
1. Brain 2. Heart 3. Kidney 4. Spleen 5. Liver 6. Colon 7. Lung 8. Small intestine  
 9. Muscle 10. Stomach 11. Testis 12. Placenta 13. Pituitary 14. Thyroid  
 15. Adrenal Gland 16. Pancreas 17. Ovary 18. Uterus 19. Prostate 20. PBL  
 21. Fetal Brain 22. Fetal Liver 23. Fat 24. Mammary Gland

**Figure 3: Sure-Race Mouse Panel (two 48-well plates)**



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. Adenal Gland  
 14. Salivary Gland 15. Uterus 16. Prostate 17. Embryo 8-9 day 18. Embryo 9.5 day  
 19. Embryo 12.5 day 20. Embryo 19 day 21. Breast/Virgin 22. Breast/Pregnant  
 23. Breast/lactating 24. Breast/Involuting

**Figure 4. Schematic View of the RACE Procedure Using the Sure-RACE™ Panel.**





### First-Round PCR Analysis:

1. Prepare the PCR premix in a 1.5 mL microcentrifuge tube as follows (for a 48-well panel):

| <i>Stock solution</i>                   | <i>Volume (uL)</i> |
|---|--------------------|
| dH <sub>2</sub> O (sterile)             | 835                |
| 10X PCR buffer (with Mg <sup>2+</sup> ) | 100                |
| dNTP mix (10 mM each)                   | 20                 |
| ADP1 (10 uM)                            | 20                 |
| GSP1 (10 uM)                            | 20                 |
| Taq DNA polymerase (5U/uL)              | 5*                 |
| <b>Total</b>                            | <b>1000</b>        |

\*Add Taq DNA polymerase just before use in Step 3. Mix by pipetting.

2. Remove the Sure-RACE™ plate from the -20°C freezer, peel back the sealing film, and place on ice immediately.
3. Add 20 uL of the PCR premix to each well. Pipet the premix to the side wall of each well to avoid cross-contamination (the dried cDNAs are at the very bottom of each well).
4. Seal the top of the RACE panel with a new adhesive cover sheet, making sure that each well is tightly sealed.
5. Transfer the RACE panel from ice to the block of the thermal cycler heating block which has been pre-equilibrated at 94°C. Make sure that each well has a tight contact with the heating block. Tighten the lid to ensure a direct contact between the heated lid and sealing film.
6. Perform the PCR using the following thermal cycling parameters:
  - I. 94°C for 3 minutes
  - II. 10 cycles of:
    - 94°C for 30 seconds
    - 68°C for 30 seconds
    - 72°C for 2~4 minutes
  - III. 15 cycles of:
    - 94°C for 30 seconds
    - 62°C for 30 seconds
    - 72°C for 2~4 minutes
  - IV. 72°C for 6 minutes
  - V. Hold at 4°C

## Second-Round PCR Analysis:

7. Set up the nested PCR premix on ice, as follows (for a 48-well plate):

| <i>Stock Solution</i>       | <i>Volume (uL)</i> |
|-----------------------------|--------------------|
| dH <sub>2</sub> O (sterile) | 785                |
| 10X PCR buffer              | 100                |
| dNTP mix (10 mM each)       | 20                 |
| ADP2 (10 uM)                | 20                 |
| GSP2 (10 uM)                | 20                 |
| Taq DNA polymerase (5U/uL)  | 5*                 |
| <b>Total</b>                | <b>950</b>         |

\*Add Taq DNA polymerase just before use in Step 9. Mix by pipetting.

8. Remove the PCR panel from the thermal cycler after the program has finished in Step 6. Remove the sealing film, add 180 uL dH<sub>2</sub>O to each well and mix gently. Pipet 1 uL of the diluted template into a new 48-tube PCR panel (not supplied in the kit). Keep the plate on ice.
9. Aliquot 19 uL of the nested PCR premix to each well. Centrifuge or tap the solution to the bottom of each well. Seal the tube and leave the panel on ice.
10. Transfer the panel from ice to the thermal cycler which has been equilibrated to 94°C.
11. Perform the PCR using the following thermal cycling parameters:
  - I. 94°C for 3 minutes
  - II. 5 cycles of:
    - 94°C for 30 seconds
    - 65°C for 30 seconds
    - 72°C for 2~4 minutes
  - III. 5 cycles of:
    - 94°C for 30 seconds
    - 62°C for 30 seconds
    - 72°C for 2~4 minutes
  - IV. 25 cycles of:
    - 94°C for 30 seconds
    - 60°C for 30 seconds
    - 72°C for 2~4 minutes
  - V. 72°C for 6 minutes
  - VI. Hold at 4°C
12. Add 4 uL of 6X DNA loading dye to each well after PCR, mix gently by slow vortexing.
13. Load 16 uL of the final RACE products on a 1.2% agarose gel. Use 5 uL of OriGene DNA Quanti-Ladder™ (provided) per well as size markers. Since multiple PCR products may appear and have to be separately extracted for direct sequencing, run the gel long enough to separate bands. A long-wavelength UV source (300 nm) is recommended for visualization of bands to minimize DNA nicking.

# TROUBLE-SHOOTING GUIDE

## **The RACE products appear as multiple bands in one or several tissues.**

- As described earlier, multiple bands may be the result of differential RNA initiation or splicing at the 5' end, which may or may not be tissue-specific. In most cases, differential initiation and splicing can be determined when bands of the same size are seen in more than one tissue, and the band pattern can be reproduced when a separate RACE analysis is performed on the duplicate plate using the same set of primers. Occasionally, multiple bands may appear for a gene where alternate transcriptional initiation or splicing is not expected, especially for very long transcripts. If the gene of interest is expressed in several or most tissues, it should be possible to identify one single major band of the same size from different tissues. The minor bands usually represent incomplete cDNAs (truncation products), and are usually not reproducible in a duplicate experiment. It is strongly suggested that sequencing of the major common band that is observed in different tissues be performed. If the sequencing results suggest it is a false-positive, the following are suggested:
- Design another gene-specific primer further downstream of GSP1. Perform the first-round PCR with the new primer. For the second-round PCR, set up two separate nested PCR reactions using the original GSP1 and GSP2 primers, respectively. Three sets of RACE experiments will enable you to determine which band is the specific product.
- Optimize the PCR parameters by changing the annealing temperature, cycle numbers, annealing time,  $MgCl_2$  concentration, or using the hot-start technique.
- Change the RACE strategy by performing the first-round PCR for 35 cycles and second-round (nested) PCR for 20 cycles. The specificity of the two gene-specific primers could be different, so a change of relative numbers could improve the specificity of the amplification reaction.

## **The RACE products appear as a smear or have a very heavy background.**

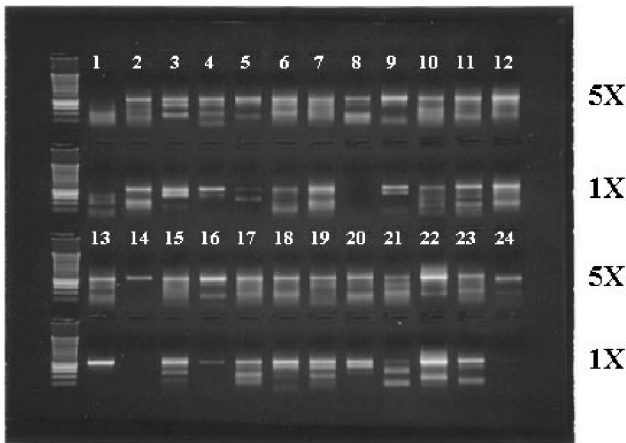
- With 24 tissue cDNAs on the panel, and two dilutions for each cDNA, discrete bands should always be detected in some of the tissues. In the case where your cDNA is expressed only in one or two tissues, and the final RACE product is a smear, it is most likely caused by the inclusion of too much PCR products from the first amplification. Suggested solutions are:
- Make further dilutions of the original cDNAs for first round PCR
- Perform long PCR if the missing portion is over 3~5 kb
- Dilute the first round PCR product to 1:100 (or 1:1000), instead of 1:10
- Reduce the second-round (nested) PCR cycle number
- Perform PCR thereafter only for identified specific tissue(s)

**No bands could be identified after the nested PCR analysis.**

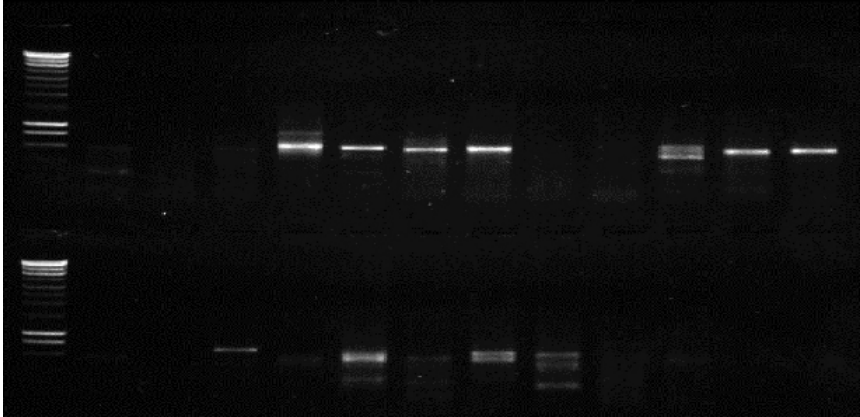
- The most likely causes for no identifiable bands are the two gene-specific primers and/or the selected PCR conditions. Suggested solutions are:
- Perform positive controls using the TRP1 and TRP2 primers provided. This will determine if the PCR components and parameters are correct.
- Increase the cycle number of the second-round PCR to 40, in case your transcript is extremely rare (low abundance).
- Lower the annealing temperature
- Redesign two gene-specific primers, and ensure these two primers are compatible with the two adapter primers, ADP1 and ADP2.

## EXAMPLES

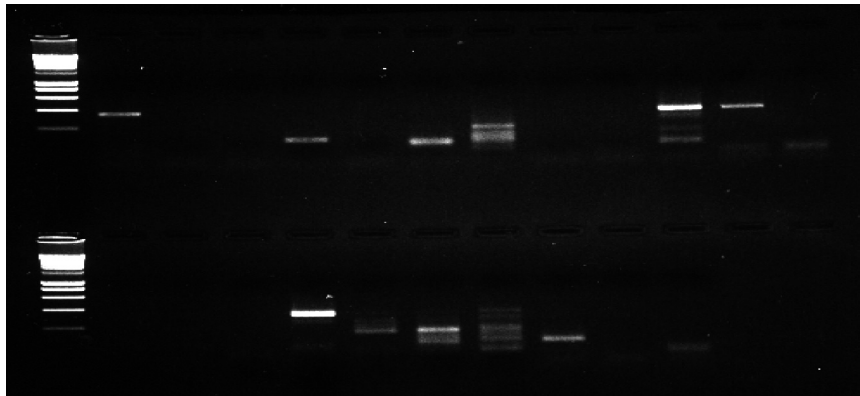
**Figure 5: 5' RACE of human transferrin receptor gene (5kb) using Sure-Race human panel. PCR conditions are as described in this protocol.**



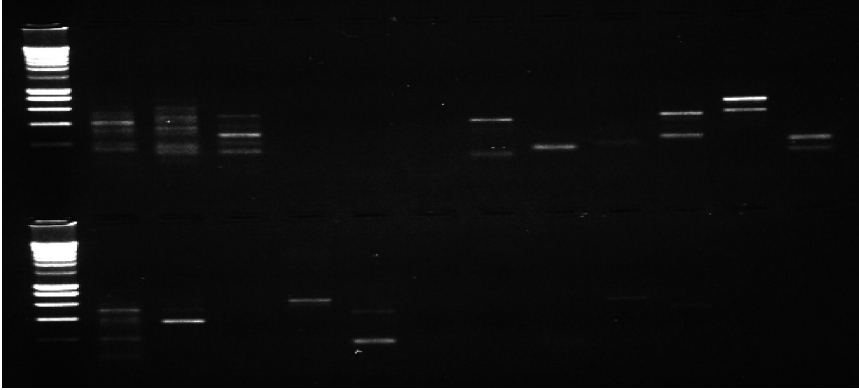
**Figure 6.** To complete the 5'-end sequences of long and rare transcripts with extensive secondary structure. The ataxia telangiectasia (ATM) gene encodes a ubiquitous 12 kb transcript with extensive confirmations and heterogeneous 5' end.



**Figure 7.** To discover alternate RNA start-sites derived from different tissue-specific transcriptional promoters. The intercellular adhesion molecule-1 (ICAM-1) gene uses different promoters to initiate transcription.



**Figure 8. To identify differentially-spliced transcripts that contain alternate 5' exons that are utilized in different tissues. The fibroblast growth factor 1 (FGF-1) gene makes use of different 5' exons to regulate its expression.**



## FREQUENTLY ASKED QUESTIONS

**What is the advantage of performing a 24-tissue RACE analysis if the tissue site(s) of expression of my gene is known?**

By surveying more tissues where your gene is expressed, you markedly increase the probability of successfully completing the 5' end of your transcript of interest. Each cDNA synthesis yields a different proportion of full-length reverse transcripts because of differences in the intactness of the starting mRNA, in relative mRNA accumulation between tissues, and in the efficiency of cDNA synthesis between RNA populations. Additionally, the appearance of an identical PCR product in different tissues assures specificity of the RACE analysis.

**Can I use the Sure-RACE™ panel to study 5' alternate RNA splicing in different tissues?**

Yes. The 24-tissue RACE panel was specifically designed not only for standard RACE analysis, but also for studying 5'-end differential splicing and alternate RNA start-sites. Tissue-specific alternate transcriptional initiation sites, alternate intron splicing and exon usage, can be determined by using the Sure-RACE™ panel with just two gene-specific primers.

**Can I use the RACE panel to determine the sites of expression of my gene?**

We do not recommend using these panels to study tissue distribution of expression of your gene. The amount of cDNA has not been “normalized” between the tissues. As such, it can only give you a rough idea of where your gene is expressed but not the relative levels of expression between the various tissues. OriGene’s Rapid-Scan™ Gene Expression Panels are designed specifically for defining gene expression profiles.

**Can the Sure-RACE™ panel be used for 5’ end to 3’ end PCR amplification to generate a full-length clone?**

Yes. These cDNAs may be used for long cDNA cloning because of the strategy that was used for their synthesis. Full-length cDNAs as long as 13-kb in length have been detected in testing. To generate long PCR products, you may have to choose the appropriate thermostable DNA polymerase (e.g. Taq) and prolong the extension time.

**Can I use the RACE products for direct sequencing?**

Yes. Individual bands can be excised from the agarose gel, and the DNA can be extracted and used directly for sequencing (QIAquick PCR purification kit). Your own GSP2 primer is a better choice for direct sequencing to avoid different truncations at 5’ when ADP2 is used. If the amount of PCR product is insufficient, excised band(s) can be cut out and re-amplified with the ADP2 and GSP2 primers. Meanwhile, the DNA bands can also be cloned into a TA cloning vector for sequencing.

**How do I choose the right band for sequencing if multiple components appear in the same or different tissues after the RACE reaction?**

There are several scenarios to consider. The first is that no differential splicing occurs at the 5’ end of your RNA. If your gene is expressed in more than one tissue, you should choose the largest common band from these tissues. Always choose well-separated bands for sequencing. The second scenario is that alternate transcriptional initiation or splicing exists at the 5’ end of the RNA, either in the same tissue or in different tissues. Comparison of different size bands in all the tissues will enable you to determine how many alternatively initiated or spliced transcripts exist. While truncated PCR products may exist in some tissues, differentially initiated or spliced transcripts have defined sizes and they can be found frequently in more than one tissue. To confirm the existence of alternatively initiated or spliced products, duplicate RACE reactions should be conducted using the four plates that are provided.

**Can I use the RACE panel to isolate a full-length clone if I have a 3'-end EST sequence?**

Yes. RACE is the quickest approach to obtain 5' end information based on the availability of a short 3' end sequence. If the cDNA is under 5 kb in length, you usually can have the full length sequence from just one RACE experiment. However, if your cDNA is above 5 kb, we suggest that you use long PCR for your analysis. Alternatively, additional RACE can be conducted to complete the whole sequence based on the sequence from a previous RACE experiment.

**Can I use the Sure-RACE™ panel for 3'-end RACE?**

No. This product was specifically designed for 5'-end RACE because two unique adapter primers were specifically linked to the 5' end. Primers could be designed from the 3'-end linker sequence for 3'-end RACE, but it may not be as efficient as the 5'-end RACE.

**Can I dilute the cDNAs in the RACE panel and use them for multiple experiments?**

Two cDNA concentrations are provided to facilitate analysis of both abundant and rare transcripts. They are designated 5X for analysis of most transcripts and 1X for highly-abundant transcripts. These concentrations have been tested extensively to give a positive signal even for very rare transcripts (for example, 2 copies per million). In general, RACE analysis with a higher concentration of cDNAs gives you more consistent results. If the cDNA concentration is too low, or your primer specificity is not ideal, you may not be able to amplify specific but rare targets and may detect only nonspecific but abundant targets. We recommend that you use the concentration provided. If you know that your RNA is very abundant in a specific tissue, we suggest that you start by using the 1X concentration.

## CITATIONS

1. Selective Modulation of Integrin-mediated Cell Migration by Distinct ADAM Family Members, Jing Huang, Lance C. Bridges, and Judith M. White, *Mol. Biol. Cell*, Oct 2005; 16: 4982 - 4991.
2. Involvement of a Rac Activator, P-Rex1, in Neurotrophin-Derived Signaling and Neuronal Migration, Masato Yoshizawa, Takeshi Kawauchi, Masaki Sone, Yoshiaki V. Nishimura, Mami Terao, Kaori Chihama, Yo-ichi Nabeshima, and Mikio Hoshino, *J. Neurosci.*, Apr 2005; 25: 4406 - 4419.