Clontech Laboratories, Inc.

cDNA Panels User Manual

Cat. No(s). Many
(111511)

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I. Introduction

A. Summary

- Clontech's cDNA Panels are sets of first-strand cDNA generated from different human, mouse, and rat tissues and cell lines. Designed to suit both broad research needs and specific applications, they include **MTC (Multiple Tissue cDNA) Panels** and an **Apoptosis cDNA Panel**.
- These panels offer fast and accurate PCR analysis of gene expression across multiple tissues (using gene-specific primers) in a fraction of the time required for Northern or dot blot analysis. In addition, the higher sensitivity of RT-PCR makes it possible to detect some transcripts that cannot be detected in Northern blots, and allows you to determine differences between samples over a broader linear range.
- Our cDNA Panels are virtually free of genomic DNA, ensuring that all signals generated are from actual mRNA transcripts.

B. MTC (Multiple Tissue cDNA) cDNA Panels

- MTC Panels consist of cDNAs from poly A^+ or total RNA, isolated from a series of different tissues or cell lines. Each MTC panel has been normalized to several different housekeeping genes to ensure accurate assessment of target mRNA abundance by minimizing the small tissue-specific variations in expression of any single HK gene, as described in the Appendix.
- In addition, the Human Panels as a whole are normalized against one another, allowing comparison of results obtained using different human panels.
- When comparing gene expression in divergent tissues with PCR, the use of normalized cDNA permits more accurate assessment of tissue specificity and relative abundance of target mRNAs than does PCR using nonnormalized cDNA (Spanakis & Brouty-Boyé, 1994; Spanakis, 1993; Liew *et al*., 1994; Savonet *et al*., 1997). Thus, variations in band intensity observed with your PCR products may be more reliably attributed to true differences in target mRNA abundance as long as the PCR is performed under nonsaturating conditions. However, to obtain truly quantitative data, you must use internal PCR standards and more rigorous data analysis methods (Siebert & Larrick, 1993).

C. Apoptosis cDNA Panel

- For rapid screening of gene expression under different apoptotic conditions, we provide an Apoptosis cDNA panel, the **MCF7 cDNA Panel**. This panel, designed for studies focusing on p53-regulated apoptosis, contains cDNA from modified and unmodified MCF7 cells expressing dominant-negative p53. It consists of first-strand cDNA samples synthesized from the total RNA of MCF7 cells that were subjected to several different apoptosis-inducing treatments and/or treatment durations.
- Since each sample in the Apoptosis cDNA Panel is derived from the same cell line (unlike MTC Panels, which consist of cDNA samples from different tissues), normalization based on housekeeping gene expression is not critical and the cDNAs in these panels are provided at a fixed DNA concentration.

D. PCR Screening of cDNA Panels

- PCR screening of cDNA Panels allows detection of mRNAs of all abundance levels, including very rare transcripts (Siebert & Huang, 1997; Ruddy, 1997). Results are obtained in only a few hours, many times faster than hybridization analyses.
- For successful PCR screening, you must have enough sequence information to design appropriate PCR primers, and the PCR conditions should be optimized for each new set of primers.
- For best results with your cDNA Panel, we recommend using Titanium® *Taq* DNA Polymerase (Cat. No. 639208).

E. Positive Control

- Each cDNA Panel includes 5' and 3' control PCR primers that amplify a ~1-kb fragment of the G3PDH gene in each of the cDNA preparations—for verifying cDNA quality/reaction set-up effectiveness.
- A control cDNA template is also provided for optimizing cycling parameters; please note that the control cDNA has not been normalized to the rest of the panel.

II. List of Components

Please refer to the Certificate of Analysis (CofA) accompanying each cDNA Panel for detailed tissue/cell line source information. Each cDNA preparation is sufficient for 10 PCR reactions of 50 µl each. The control PCR primers and control cDNA are also sufficient for 10 reactions.

Store all components at –20°C.

All cDNA Panels:

- 50 µl **Control cDNA**

  NOTE: The control cDNA has not been normalized to the rest of the panel.

- 40 µl **G3PDH Control Primer Mix** (10 µM each)

  For the Human cDNA Panels, the control primers are the Human G3PDH Control Amplimer Set:
  5' (upstream) primer: 5'–TGAAGGTCGGAGTCAACGGATTTGGT–3'
  3' (downstream) primer: 5'–CATGTGGGCCATGAGGTCCACCAC–3'

  For the Mouse cDNA Panels, the control primers are the Mouse G3PDH Control Amplimer Set:
  5' (upstream) primer: 5'–TGAAGGTCGGTGTGAACGGATTTGGC–3'
  3' (downstream) primer: 5'–CATGTAGGCCATGAGGTCCACCAC–3'

  For the Rat cDNA Panels, the control primers are the Rat G3PDH Control Amplimer Set:
  5' (upstream) primer: 5'–TGAAGGTCGGTGTCAACGGATTTGGC–3'
  3' (downstream) primer: 5'–CATGTAGGCCATGAGGTCCACCAC–3'

III. Additional Materials Required

The following reagents are required but not supplied:

- **Gene-specific PCR primers**
  Appropriate 5' and 3' primer pairs to amplify your gene of interest. See Section IV.B for primer design tips. Alternatively, RT-PCR primers are available from Clontech.
- **PCR-grade deionized H_2O** (filter-sterilized)
  (Do not use autoclaved H_2O for PCR. The recycled steam in some autoclaves can introduce salts and other contaminants that may interfere with PCR reactions.)
- **Mineral oil** (Sigma No. M-3516)
- **0.5 ml PCR reaction tubes** (Applied Biosystems GeneAmp 0.5 ml reaction tubes, Cat. No. N8010737 or N8010180)
- **Thermal cycler** (Perkin-Elmer DNA Thermal Cycler 480 or equivalent)
- **Dedicated pipettors**
- **PCR pipette tips** with hydrophobic filters. **Do not autoclave pipette tips.**
- **DNA size markers** (see Section IV.C.7)
- **5X Stop/loading buffer** (see Sambrook & Russell [2001] for recipes)
- **Titanium *Taq* PCR Kit**
  The Titanium *Taq* PCR Kit (Cat. No. 639210) includes 50X Titanium *Taq* DNA Polymerase, 10X Titanium *Taq* PCR Buffer, 50X dNTP Mix (10 mM each), control DNA template, control primer mix, and PCR-Grade Water. If you do not use the Titanium *Taq* PCR Kit, see Table I.

IV. cDNA Panels PCR Protocol

A. General Considerations

1. **Thermal cycler:** The cycling parameters in this protocol have been optimized using a PE Biosystems DNA Thermal Cycler 480.
2. **Thermostable DNA polymerase**
   a. The cDNA Panel PCR protocol was optimized using Titanium *Taq* DNA Polymerase, which was developed for accurate, sensitive, and robust PCR amplification of cDNA .
   b. Hot start is automatic when you use Titanium *Taq* DNA Polymerase because the mix already contains TaqStart® Antibody. Therefore, do not use a manual hot start or wax-bead-based hot start when using Titanium *Taq* DNA Polymerase.
3. **Use of additives**
   a. If your target sequence has a high GC content (up to 90%), the PCR results can generally be improved using the Advantage GC 2 Polymerase Mix (Cat. No. 639114), which is supplied with an additive, GC-Melt Reagent—so there is generally no need to use DMSO or other additives.
   b. The addition of 2–5% DMSO will not interfere with TaqStart function and may improve results in some instances—but adding formamide or other cosolvents may disrupt TaqStart function.
   c. Excessive glycerol, solutes (e.g., salts), pH extremes, or other deviations from the recommended reaction conditions may reduce the effectiveness of TaqStart Antibody and/or *Taq* DNA Polymerase.
4. **Touchdown PCR:** We have found that "touchdown" PCR significantly improves the specificity of many PCR reactions in a wide variety of applications (Don *et al.*, 1991; Roux, 1995). See Section V.B for more information.

B. Good PCR Practices

1. **Use dedicated pipettors in a dedicated work space** to avoid nonspecific amplification.
   a. Use small aliquots of starting material to avoid contaminating your stocks.
   b. Wear gloves and use PCR pipette tips with hydrophobic filters and dedicated solutions.
2. **Careful pipetting of small volumes** is necessary to avoid tube-to-tube variation.
   a. Always be sure that there is no extra solution on the outside of the pipette tip before transfer.
   b. When adding solution to a tube, immerse the tip into the reaction mixture and deliver the contents from the pipette tip into the mixture, then rinse the tip by pipetting up and down several times.
3. **Use a Master Mix**, which contains the appropriate volumes of all reagents required for multiple PCR reactions, saves time and greatly reduces tube-to-tube variation.
   a. If multiple templates are being tested with the same primers, include primers in the Master Mix.
   b. If one template is being tested with multiple primer sets, include the template in the Master Mix.
   c. If you are setting up several sets of parallel samples, assemble multiple Master Mixes (e.g., each with a different set of primers).
   d. The Master Mix should be **thoroughly mixed before use (i.e., vortexed without bubbling).**
4. **PCR Control Reactions:** include positive and negative controls (i.e., H_2O instead of DNA template).

C. Primer Design and Target Sequences

Primer design is the single largest variable in PCR applications and the single most important factor in determining the success or failure of PCR reactions. The following general guidelines are recommended:

1. Primer Design

Important: Always check and recheck your primer design before constructing or ordering primers.

- **Length:** Primers should have a T_m of ~70°C to achieve optimal results in a two-step cycling program with a 68°C combined annealing/extension step. Therefore, whenever possible, primers should be **at least 24 nucleotides** (nt) long (25–30-mers are preferred)
- **GC Content:** Primers should have a GC content of 45–60%. Furthermore, the 3' ends of each primer should not be complementary to each other and should have a low GC content (less than three consecutive G's or C's within the first five nucleotides of the 3' end)

2. Choosing the Target Sequence

- For best results, design your gene-specific primers to amplify a target sequence of ≤1 kb (ideally, 300–800 bp). (The PCR protocol was optimized with a ~1-kb control G3PDH target sequence.)

  NOTE: With amplicons >1 kb, you need to use a longer extension time (see Section V.A) that may not be compatible with your *Taq* polymerase. In addition, targets >1 kb may result in a weak band due to competition with smaller, nonspecific products, especially when using >35 cycles.

- For cDNA's larger than ~2.5 kb, design the primers to anneal within the 3' portion of the cDNA.
- To reduce the risk of amplifying a related member of the same gene family, design at least one of the primers to anneal within the 3' UTR (untranslated region) if possible.
- Our cDNA Panels are prepared from purified poly A^+ or total RNA that are virtually free of genomic DNA. Thus, you do not have to worry about introns when designing your primers.

3. Template Quality

- Panel cDNAs range in size from 0.1 to at least 6 kb and are virtually free of genomic DNA.
- The DNAse used to treat RNA samples is fully removed before first-strand cDNA synthesis.

NOTE: Some depurination occurs when the cDNA is denatured during thermal cycling, and therefore, the denaturation time should be kept as short as possible (30 sec in this protocol).

D. PCR Protocol

1. Design gene-specific primers using the guidelines in Section C.
2. Obtain or prepare cDNA from a tissue in which the target gene is known to be expressed. Use this cDNA as a PCR template to test the gene-specific primers.
3. Prelabel the PCR tubes and prepare a PCR master mix for the planned number of reactions (see Tables I & II). To ensure that you have sufficient Master Mix for your experiment, calculate the volume for two reactions for each sample in the cDNA Panel (one using your gene-specific primers, and the other using the control G3PDH primers), a positive and a negative control, and 10% extra.

NOTE: If your stock solutions are a different concentration than those shown in Table I, adjust volumes accordingly, and use correspondingly less (or more) H_2O.

Table 1. Preparation of the PCR Master Mix

Reagent	Amount per reaction (µl)
PCR-grade deionized H_2O	36
10X PCR reaction buffer	5
50X dNTP mix (10 mM each)	1
50X Titanium *Taq* DNA Polymerase*	1
Total volume per rxn	43

* We recommend using Titanium *Taq* DNA Polymerase (Cat. No. 639208), although you can use *Taq* DNA polymerase alone (as per manufacturer specs). If you use *Taq* DNA polymerase alone, 3–5 more cycles will be needed in the PCR and a hot start will be required.

4. Combine 43 µl of the PCR master mix with the appropriate primer mix and cDNA sample in each prelabeled tube (Table II). For each cDNA sample in the panel, set up parallel reactions using your gene-specific primers and the G3PDH control primers, as follows:
   - **Positive Control**: use G3PDH Control Primers & Control cDNA provided with cDNA Panel.
   - **Negative Control:** use G3PDH Control Primers & 5 µl of H_2O instead of cDNA.

Table 2. Recommended PCR Set-Up

Component	Positive Control (µl)	Negative Control (µl)	G3PDH Controls (µl)	Experimental Reactions (µl)
PCR master mix	43	43	43	43
Deionized H_2O	—	5	—	—
G3PDH primers[a]	2	2	2	—
Experimental primers[a]	—	—	—	2
Control cDNA	5	—	—	—
cDNA Panel sample[b]	—	—	5	5
Total volume per rxn	50	50	50	50

[a] Includes both the 5' (upstream) and 3' (downstream) primer, 10 µM each; final concentration in the reaction mixture is 0.4 µM each.

[b] Use a separate tube for each individual cDNA preparation.

5. If your thermal cycler does not have a "hot lid", add 1–2 drops of mineral oil to prevent evaporation during cycling. A good "seal" of mineral oil should have a well-defined meniscus between the two phases. Cap the PCR tubes firmly.

6. PCR General Guidelines

Use the following general guidelines when setting up your PCR reactions according to the recommended parameters described in Step 7. The optimal parameters may vary with different thermal cyclers and will depend on your particular primers, templates, and other experimental variables. Refer to the Troubleshooting Guide (Section V) for suggestions on optimizing PCR conditions.

- **Measure Abundance before the PCR Reaction Plateau:** To be able to assess the relative abundance of a transcript in different tissues, the PCR products must be analyzed before the reaction reaches its plateau (observed when the intensity of the PCR product band does not double after an additional two thermal cycles).
- **Take Time Points:** The rate at which the PCR reaches saturation depends on the abundance of the particular target sequence in that tissue and the efficiency of the PCR primers—so it is safest to analyze the PCR products from all experimental reactions at several time points. For example, stop all the reactions after 22 cycles and remove 5 µl samples to run on a gel; then put the reaction mixtures back in the thermal cycler. Remove samples after 26, 30, 34 & 38 cycles.
- **Recommended Number of Cycles:**
  22 cycles with a 3 min annealing/extension time for the positive control reaction (Appendix, Figure 1 shows typical results with G3PDH control primers).
  25 cycles with a 3 min annealing/extension time for high- to medium-abundance cDNAs.
  30–35 cycles are required for medium- to low-abundance cDNAs.
  After 38 cycles, even very rare transcripts should produce a distinct PCR product.
  > **40 cycles** (i.e., overcycling) leads to problems with background and nonspecific amplification.
- **Use Two-Step Cycles** (i.e., denaturation at T1 followed by annealing and extension at T2), rather than three-step cycles (i.e., denaturation at T1 followed by annealing at T2, followed by extension at T3). For best results, the T_m of your gene-specific primers should be ≥70°C (see Section IV.C).
- **Denaturation Time:** We recommend a denaturation time no longer than 30 sec.
- **Extension Time:** A 3 min extension time should work well for most target sequences up to ~1 kb. A final extension time of 3 min is recommended to complete extension of all cDNA strands.

7. **Recommended Cycling Parameters**

   Begin thermal cycling using the following parameters (optimized for the control template and primers included in the kit), taking into account the general guidelines in Step 6.

   - 95°C 1 min
   - 22–38 cycles
     - 95°C (T_1) 30 sec
     - 68°C (T_2) 3 min
   - 68°C 3 min (final extension)
   - 15°C soak

8. Transfer 5 µl of your PCR reaction to a fresh tube and add 1 µl of 5X stop/loading buffer. Analyze your sample (s), along with suitable DNA size markers, on a 1.2% agarose/EtBr gel.

Expected results: If you are using the control cDNA template and G3PDH primers provided in the kit, the reaction should produce a single major fragment of 938 bp (see the Appendix, Figure 1 for typical results) when using the positive control reagents provided in the kit—and no bands in the negative (i.e., no DNA template) control.

V. Troubleshooting Guide

A. No Product Observed (or Only Weak Product Band)

Description of Problem	Solution
Too few cycles	If you have used less than 38 cycles, continue cycling and remove 5 µl samples for analysis after each additional set of four cycles (Section IV.D.6). If you used 38 cycles, try two more cycles. Do not use more than 40 cycles or you will run into problems with overcycling—i.e., you may observe multiple and/or smeared products (Sections V.B & C).
PCR component is missing or degraded	Use a checklist when assembling reactions. Always perform a positive control to ensure that each component is functional. If the positive control does not work, repeat the positive control only. If the positive control still does not work, repeat again replacing individual components to identify the faulty reagent.
Inefficient enzyme was used for PCR	If you are not already doing so, we strongly recommend that you use Titanium *Taq* DNA Polymerase, or at least compare this mix to your current enzyme.
Annealing/extension temperature is too high	Decrease the annealing temperature by increments of 2–4°C.
Suboptimal primer design	Redesign your primer(s) after confirming the accuracy of the sequence information. If the original primer(s) was less than 24 nucleotides long, try using a longer primer. If the original primer(s) had a GC content of less than 45%, try to design a primer with a GC content of 45–60%.
Target sequence is too large	Check the annealing sites of the primers to make sure that the target sequence is not >1 kb. If you cannot avoid a target sequence >1 kb, add 1 min for each kb over 1 kb to the 3 min annealing/extension time (T_2) that is recommended.
Denaturation temperature is too high or too low	Optimize the denaturation temperature by decreasing or increasing it in 1°C increments. (A denaturation temperature that is too high can lead to degradation of the template, especially for long target sequences.)
Denaturation time is too short	Optimize the denaturation time by decreasing or increasing it in 10 sec increments. (A denaturation time that is too long can lead to degradation of the template, especially for long target sequences.)
Extension time is too short	(Especially with longer templates) Increase extension time in 1 min increments.
Too little enzyme	[Titanium *Taq* DNA Polymerase] is 50X for most applications. Therefore, try to optimize the cycle parameters as described above before increasing the enzyme concentration. In rare cases, yields can be improved by increasing the concentration of the enzyme mix. However, increasing the concentration more than 2X is likely to lead to higher background levels. If you are using *Taq* polymerase from another commercial supplier, follow their recommendations.
[$Mg2^+$] is too low	Titanium *Taq* DNA Polymerase performs well at a broad range of Mg^{2+} concentrations. Therefore, as long as you use the buffer included with the mix and a final concentration of 0.2 mM of each dNTP, it is unlikely that a lack of product is due to problems with the Mg^{2+} concentration. However, high concentrations of EDTA or other metal chelators in the template stock solution can reduce the effective concentration of Mg^{2+} to below a minimum level.
[dNTPs] is too low	Use 0.2 mM of each dNTP for optimal results with a wide range of applications. (This is the final concentration obtained when the 50X dNTP mix provided with the Titanium *Taq* PCR Kits is used as recommended.) Some *Taq* polymerase manufacturers recommend using concentrations higher than 0.2 mM for each dNTP when amplifying large templates. However, we have had no trouble amplifying large templates using 0.2 mM for each dNTP. Note that if you increase the concentration of dNTPs, you will also need to increase the [Mg^{2+}] proportionately.
Difficult target	Some targets are inherently difficult to amplify. In most cases, this is due to an unusually high GC content and/or secondary structure. For difficult targets, we recommend the Advantage GC 2 Polymerase Mix or Kit.

B. Multiple Products

Description of Problem	Solution
Annealing temperature is too low	Increase the annealing/extension temperature in increments of 2°C.
Touchdown PCR is needed	"Touchdown" PCR significantly improves the specificity of many PCR reactions in various applications (Don *et al.*, 1991; Roux, 1995). It involves using an annealing/extension temperature that is several degrees *higher* than the T_m of the primers during the initial PCR cycles. The annealing/extension temperature is then reduced to the primer T_m for the remaining PCR cycles. This change can be implemented in either a single step or in increments over several cycles.
Too many cycles	The PCR products should be analyzed before the reaction reaches its plateau. (Generally, if the intensity of the band does not double after an additional two cycles, the reaction has probably become saturated.) Repeat the experiment using fewer cycles. Ideally, start taking samples after 22 cycles and after each additional 4 cycles. Reducing the cycle number may eliminate nonspecific bands. In any case, do not use more than 40 cycles.
Suboptimal primer design	Redesign your primer(s) after confirming the accuracy of the sequence information. If the original primer(s) was less than 24 nucleotides long, design a longer primer. If the original primer(s) had a GC content of less than 45%, try to design a primer with a GC content of 45–60%.
Contamination	See Section V.D.

C. Products are Smeared

Description of Problem	Solution
Too many cycles	Reducing the cycle number by 3–5 cycles may eliminate nonspecific bands.
Denaturation temperature is too low	Try increasing the denaturation temperature in increments of 1°C.
Extension time is too long	Decrease the extension time in 1–2 min increments.
Poor template quality	Check template integrity by electrophoresis on a denaturing agarose gel. Repurify your template if necessary.
Touchdown PCR needed	See Section V.B.
Too much enzyme	Titanium *Taq* DNA Polymerase is 50X for most applications; however, a 1X final concentration of the enzyme mix may be too high for some applications. If smearing is observed, first try optimizing the cycle parameters as described above, then try reducing the enzyme concentration to 0.5–0.2X.
Too much template	Try a lower concentration of DNA template in the PCR reaction.
Contamination	See Section V.D.

D. Dealing with Contamination

Contamination most often results in extra bands or smearing. It is important to include a negative control in every PCR experiment (that replaces the DNA template with PCR-grade H_2O but still include the primers) to determine if the PCR reagents, pipettors, or PCR reaction tubes are contaminated with previously amplified targets.

- If possible, set up the PCR reaction and perform the post-PCR analysis in separate laboratory areas with separate sets of pipettors.
- Laboratory benches and pipettor shafts can be decontaminated by depurination. Wipe surfaces with 1N HCl followed by 1N NaOH. Then neutralize with a neutral buffer (e.g., Tris or PBS) & rinse with ddH_2O.
- We advise using commercially available aerosol-free pipette tips.
- An enzymatic method has been published for destroying PCR product carryover (Longo *et al*., 1990). It involves incorporation of dUTP into the PCR products and subsequent hydrolysis with uracil-N-glycosylase (UNG).

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VII. Appendix : MTC Panel Normalization Procedure

A. What is Normalization?

"Normalization" refers to Clontech's method for using the mRNA expression levels of several housekeeping (HK) genes to standardize the amount of cDNA present within each MTC Panel (Table III). Since it accounts for differences in transcription levels, normalization allows you to use MTC Panels to estimate the relative abundance of your target gene transcript in different tissues, when PCR is performed under nonsaturating conditions.

NOTE: Apoptosis cDNA Panels are not normalized.

B. Why is Normalization Necessary?

1. The Fraction of HK Transcripts is Smaller in Tissues with High Transcription Levels

In some tissues, such as brain, the overall level of transcription is relatively low, producing the mRNAs necessary for basal metabolism (i.e., HK gene transcripts) and a small quantity of other mRNAs. In contrast, other tissues, such as glands, are transcriptional powerhouses, producing large quantities of other mRNAs along with HK transcripts. At the same time, the level of expression of HK genes is relatively constant for all tissues (Adams *et al.*, 1993; Adams *et al.*, 1995; Liew *et al.*, 1994). Thus, the fraction of HK gene transcripts in total poly A^+ RNA is generally much lower in tissues with high overall levels of transcription (such as the pancreas) than in transcriptionally less active tissues.

2. Differences in Transcription Levels Make it Difficult to Quantify Gene Expression

RNA dot blots and Northern blots are often standardized by loading equal gram amounts of poly A^+ RNA on each dot. However, this method is not ideal for quantifying gene expression, because it does not reflect differences between tissues in overall transcriptional activity. If equal gram amounts of RNA are loaded on a membrane, dots from transcriptionally active tissues will contain fewer HK gene transcripts, while dots from transcriptionally less active tissues will contain disproportionately more HK mRNAs because there are fewer tissue-specific transcripts. Furthermore, although the expression of HK genes is relatively constant between different tissues and developmental stages, every HK gene displays some genuine tissue- and stage-specific variation (Spanakis & Brouty-Boyé, 1994; Spanakis, 1993; Liew *et al.*, 1994; Adams *et al.*, 1993; Adams *et al.*, 1995).

3. Normalization Allows Comparison of Specific Transcript Levels in Different Tissues

To account for differences in transcription levels, we developed a normalization procedure for the MTC Panel cDNAs, so they can be used to study the relative levels of specific transcripts in different tissues. The genes used for normalizing the MTC Panel cDNAs are listed in Table III. After extensive testing of 20 HK genes, these genes were chosen because they show minimal variation and belong to different functional classes (Liew *et al.*, 1994; Adams *et al.*, 1995). Normalization to these standards helps compensate for the minor tissue-specific variations in expression of any single HK gene. In those cases where a specific HK transcript level was exceptionally high or low in a particular tissue, its value was excluded from the normalization procedure (Appendix, Section C).

Table 3. Housekeeping Genes Used to Normalize MTC Panels

HK Gene	Used for Human Panels	Used for Mouse & Rat Panels
α-tubulin	yes	no
ß-actin	yes	yes
glyceraldehyde-3-phosphate dehydrogenase (G3PDH)	yes	yes
phospholipase A2	yes	yes
ribosomal protein S29	no	yes

C. PCR Analysis of Gene Expression in Normalized MTC Panels

1. An equal amount of cDNA from each individual preparation was used as a template in PCR with primers for each of the four HK genes.
2. The PCR reactions were stopped before they reached their plateau, and equal volumes of the reaction mixtures were electrophoresed on ethidium bromide/agarose gels (Figure 1).
3. The relative intensity of each PCR product band was determined by video imaging and computer analysis, and an "average" band intensity was determined. If necessary, the concentration of individual cDNA preparations was then adjusted so that the average band intensity for the HK genes used to normalize the panel varied by no more than 20 percent.
4. If the expression of a certain HK in a particular tissue was repeatedly found to be markedly higher or lower than in the other tissues, it was assumed that this reflects tissue-specific up- or down-regulation of that gene; such tissue-specific regulation has been reported for many HK genes (Spanakis & Brouty-Boyé, 1994; Spanakis, 1993; Liew *et al.*, 1994; Adams *et al.*, 1993; Adams *et al.*, 1995). Thus, to avoid skewing the normalization, these unexpectedly high or low data points were omitted when calculating the average band intensity of that HK gene within the panel.
    a. For example, the level of G3PDH in skeletal muscle was exceptionally high, as might be expected for an enzyme like G3PDH, possibly due to the high ATP production in this tissue. The slightly stronger PCR signal obtained for G3PDH in the reaction using normalized skeletal muscle cDNA (Figure 1, Panels A & B, Lanes 6) is consistent with the results obtained using Northern blots.
    b. Likewise, the slightly weaker PCR signal obtained for G3PDH in the reaction using normalized pancreas cDNA (Figure 1, Panel A, Lane 8) is consistent with what is expected for this hormonal tissue, which is producing massive amounts of preproinsulin and other specialized proteins, and relatively small amounts of G3PDH.

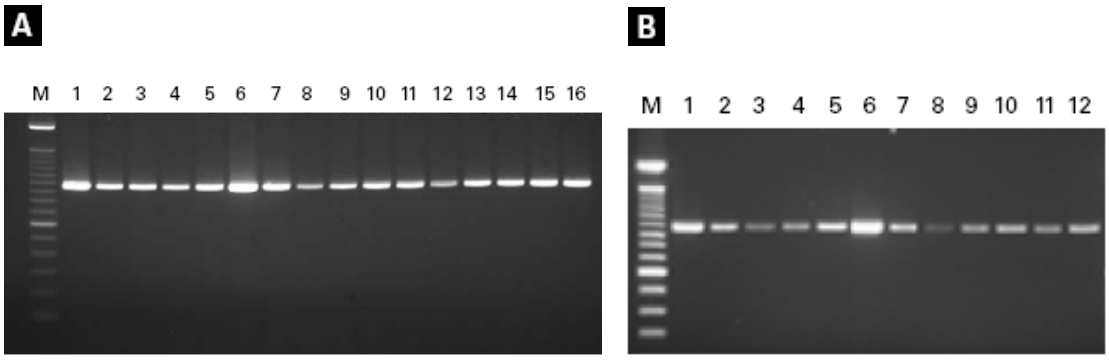

Figure 1. Comparison of tissue expression pattern of G3PDH mRNA in the sample MTC Panels. Each of the indicated MTC Panel cDNAs was used as a PCR template with the control G3PDH primers. After 20–22 amplification cycles, samples were electrophoresed on 2% agarose/EtBr gels. **Panel A.** Lanes 1–8. Human MTC Panel I (Cat. No. 636742); Lanes 9–16. Human MTC Panel II (Cat. No. 636743). Lane 1: heart. Lane 2: brain. Lane 3: placenta. Lane 4: lung. Lane 5: liver. Lane 6: skeletal muscle. Lane 7: kidney. Lane 8: pancreas. Lane 9: spleen. Lane 10: thymus. Lane 11: prostate. Lane 12: testis. Lane 13: ovary. Lane 14: small intestine. Lane 15: colon. Lane 16: peripheral blood leukocyte. Lane M: 100-bp DNA ladder. **Panel B.** Mouse MTC Panel (Cat. No. 636745). Lane 1: heart. Lane 2: brain. Lane 3: spleen. Lane 4: lung. Lane 5: liver. Lane 6: skeletal muscle. Lane 7: kidney. Lane 8: testis. Lane 9: 7-day embryo. Lane 10: 11-day embryo. Lane 11: 15-day embryo. Lane 12: 17-day embryo. Lane M: 100 bp DNA ladder. Note: G3PDH is known to be expressed at high levels in skeletal muscle and at low levels in testis.

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