L4H 418
A09

Chapter 59
Quantitative Competitive PCR

JAMES W. TUNG, AARON B. KANTOR, AND LEONORE A. HERZENBERG

The advent of polymerase chain reaction (PCR) technology has allowed researchers to detect RNA from small numbers of cells [1]. The exponential amplification nature of PCR permits the detection of rare messages at a much more sensitive level, sometimes more than 1000-fold, than Northern hybridization and RNase protection [2]. However, it has been difficult to obtain quantitative information from PCR, because small variations in amplification efficiency can result in large changes in product yield.

A common approach for PCR quantitation is to use a housekeeping gene as an external or internal standard [1, 3–5]. Both strategies are based on the assumption that the expression of a housekeeping gene, such as β-actin, hypoxanthine-guanosine phosphoribosyl transferase (HPRT), or β_2- microglobulin, is constant regardless of cell type and stimulation condition. Hence, a message from these housekeeping genes can theoretically serve as a control for total RNA. Housekeeping gene products have been used in two formats. As an external standard, the housekeeping gene is amplified independently of the target gene, that is, the two reactions are done in separate, but parallel, tubes. The result is normalized by comparing the PCR products of the housekeeping gene in different samples. Other researchers have also used housekeeping genes as internal controls where both the housekeeping gene and the target gene are coamplified in the same tube.

A number of problems are associated with these two approaches. First, using a housekeeping gene as an external control does not correct for tube to tube variation in PCR amplification efficiency. Second, the different PCR primers for the target gene and housekeeping gene may operate at different efficiencies during coamplification in a single tube. Additional difficulties may arise if both target and housekeeping genes are not expressed at similar levels. If the expression levels between the two genes vary greatly, the PCR amplification of one gene may reach saturation much earlier than the other, which in turn will distort the results. Furthermore, multiple sets of primers may interfere with each other during PCR amplification. Finally, the basic assumption that the expression of housekeeping genes is constant may not be appropriate in all cases. The expression of most housekeeping genes changes as cells are stimulated [6–8]. Housekeeping gene expression can also vary with viral infection as has been shown by the increased expression of the β-actin gene by human cytomegalovirus (J. Vierra, unpublished data). Similarly, different cell types may actually contain dissimilar levels of housekeeping gene expression. To overcome the above difficulties, quantitative-competitive-reverse-transcription-PCR has been used successfully for RNA quantitation.

Quantitative competitive RT-PCR

In quantitative-competitive-reverse-transcription-PCR (QC-RT-PCR or simply QC-PCR), a known amount of reference RNA template is reverse transcribed and amplified along with the target message in the same tube [9, 10]. The reference RNA template in this procedure serves as an internal control for both cDNA synthesis and PCR amplification. The reference template contains the same PCR primer binding sites as the target gene and shares much of the target sequence. Therefore, the two sets of templates compete for the same PCR primers. The reference template is designed so that its PCR product can be distinguished from the wild-type PCR product either by size or restriction enzyme sites.

The reference template is artificially created by a small insertion or deletion between the two primer sites, or by mutagenesis that either creates or eliminates a restriction site in the target cDNA sequence [11]. The reference DNA template is then *in vitro* transcribed using T7 RNA polymerase and the reference DNA is subsequently removed by digestion with RNase-free DNaseI. The resulting reference RNA is quantitated by spectrophotometer and diluted accordingly. Before cDNA synthesis and PCR amplification, the unknown amount of target RNA is added to tubes containing known amounts of the diluted reference RNA. After amplification, the PCR products are separated by electrophoresis on an agarose gel and visualized by ethidium bromide staining [11–13]. Alternatively, one can radioactively label one of the PCR primers and quantitate the radioactivity in the PCR products [11, 14, 15].

The most important feature of QC-PCR is that the relative ratio between the target RNA and reference RNA is conserved in the PCR products. During PCR amplification, both sets of templates compete equally for the available *Taq* polymerase, primers, and dNTP, thus preserving the ratio between templates. When the reference RNA and target RNA are present in equal amounts, the respective PCR products will also be present in equal amounts. Because it is the relative ratio between the PCR products, not the absolute amount, that is important for quantitation, one can still achieve accurate quantitation even if there is variability in PCR amplification efficiency among tubes. The readings obtained from ethidium bromide staining of PCR products are corrected for the size difference between the target and reference PCR products. The ratio of the two PCR products is plotted against the amount of input reference RNA template. The unknown target template is determined by interpolating the equivalence point at which the amount of the reference PCR products and the target PCR products are equal.

QC-PCR has been applied to quantitate numerous messages of biological interests. For example, QC-PCR has been used to determine HIV-1 RNA levels in patient sera and to follow HIV-1

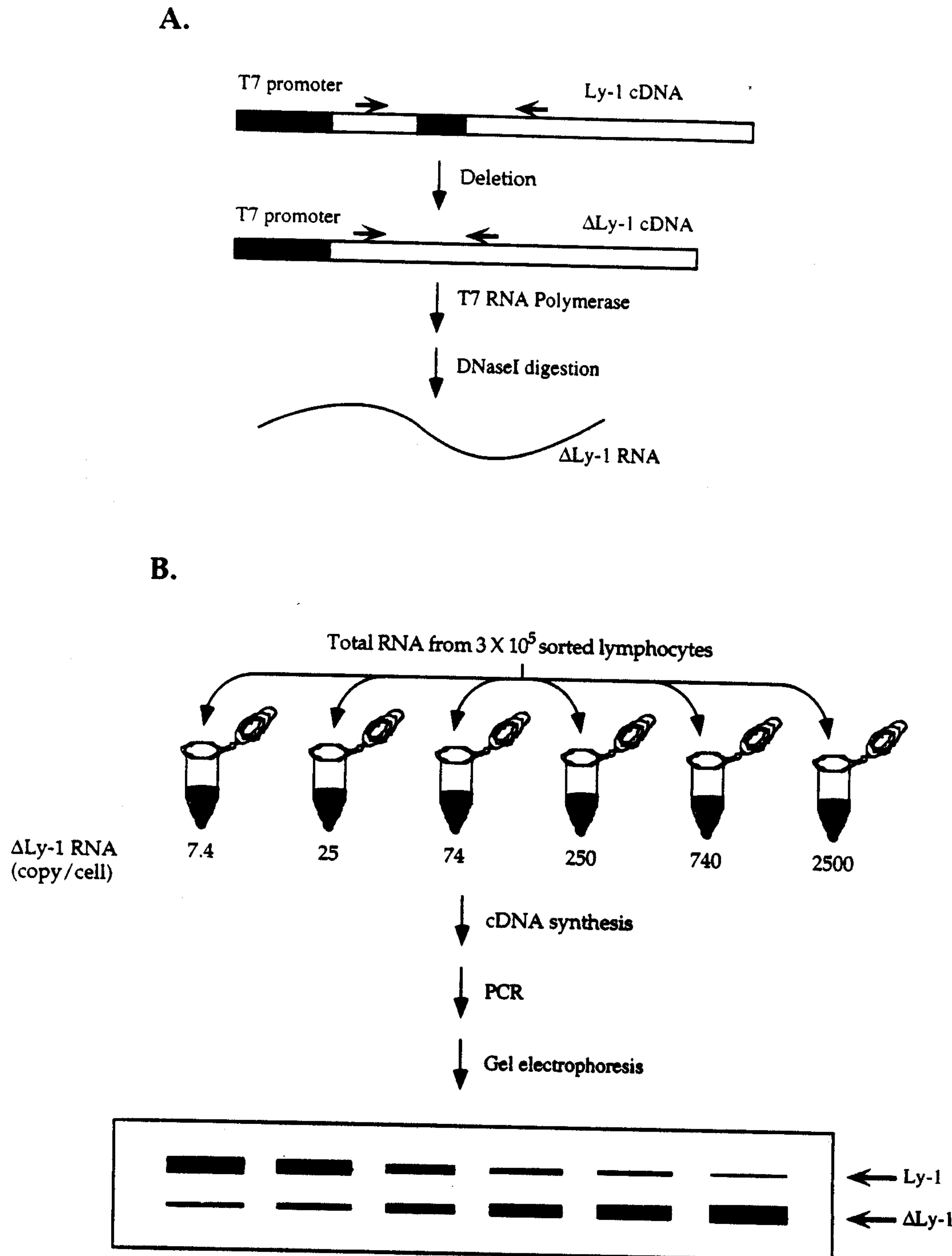
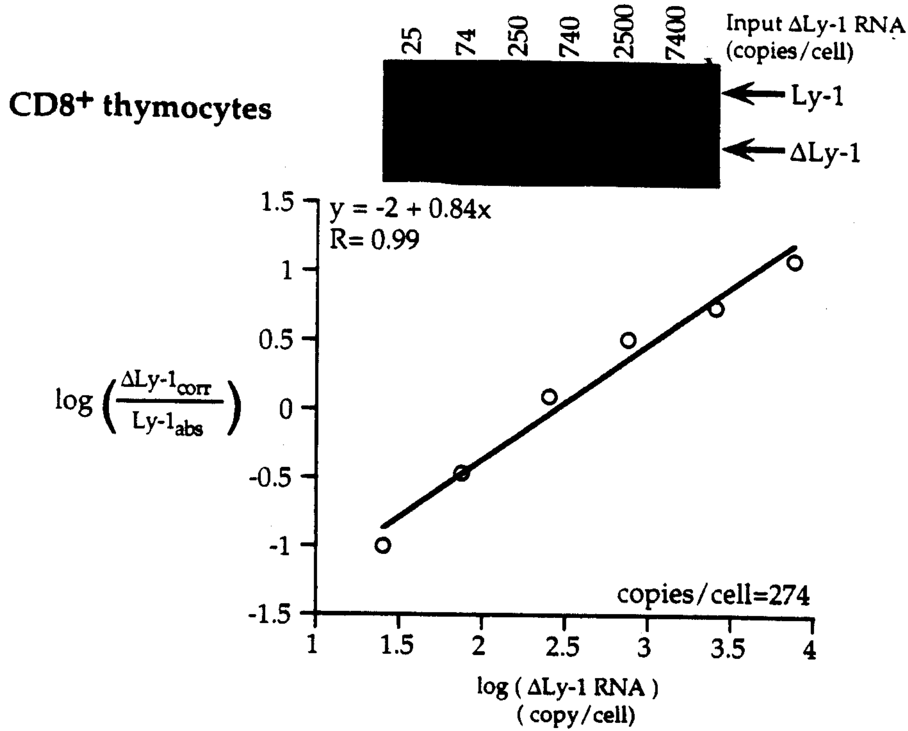


Fig. 59.1. *Steps for performing QC-PCR.* (A) Preparation of ΔLy-1 RNA. The ΔLy-1 construct was created by deleting 196 bp between two Bpu1102I sites. ΔLy-1 RNA was *in vitro* transcribed at 37°C for one hour in a final volume of 25 μl containing 40 mM Tris-HCl pH 8, 8 mM $MgCl_2$, 50 mM NaCl, 2 mM spermidine, 30 mM DTT, 400 μM rNTP, 1 μg ΔLy-1 DNA template, and 25 units of T7 RNA polymerase (Strategene, La Jolla, CA). ΔLy-1 DNA is removed by adding 10 units of RNase-free DNaseI (Boehringer Mannheim, Indianapolis, IN) to the reaction and incubate for one hour at 37°C. ΔLy-1 RNA is purified using RNAzol (Tel-Test, Inc., Friendswood, TX), a modified guanidine isothiocyanate RNA isolation method [32]. The concentration of purified ΔLy-1 RNA is determined by spectro-photometer and diluted accordingly in DEPC-treated water. The diluted ΔLy-1 RNA is stored at -70°C with no signs of degradation during the course of these experiments. (B) Total RNA was isolated from FACS-sorted populations using RNAzol. Six equal aliquots of total RNA (from the equivalent of 37,500 cells) were added to tubes containing serially diluted ΔLy-1 RNA. The RNA mixture was reverse transcribed in a final volume of 20 μl containing 50 mM Tris-HCl (pH8.3 at 25°C), 75 mM KCl, 3 mM $MgCl_2$, 8 mM DTT, 0.5 mM dNTP, 40 ng random hexamer, 1 unit RNase inhibitor (Promega, Madison, WI), and 200 units Superscript RT II (Gibco-BRL, Bethesda, MD). The cDNA synthesis reaction was incubated at 37°C for two hours and heat inactivated at 65°C for 10 min. The PCR reaction was carried out using 4 μl of cDNA reaction in a 50 μl volume containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 1.5 mM $MgCl_2$, 200 μM dNTP, 2.5 units *Taq* polymerase (Promega) and 25 pmole of Ly-1 and rcLy-1 PCR primers (Ly-1 PCR primer: 5′ TTC TGC CTC GGA CAG TCT GG 3′; rcLy-1 PCR primer: 5′ GCC TGT CCT TGG CCT TGT AG 3′). The PCR reaction was heated to 94°C for two minutes and cycled 36 to 41 times through the following steps: 94°C for 40 seconds, 62°C for 40 seconds, and 72°C for 45 seconds. Ten μl of the PCR reactions were electrophoresed on 0.75% agarose gel and visualized by ethidium bromide staining.



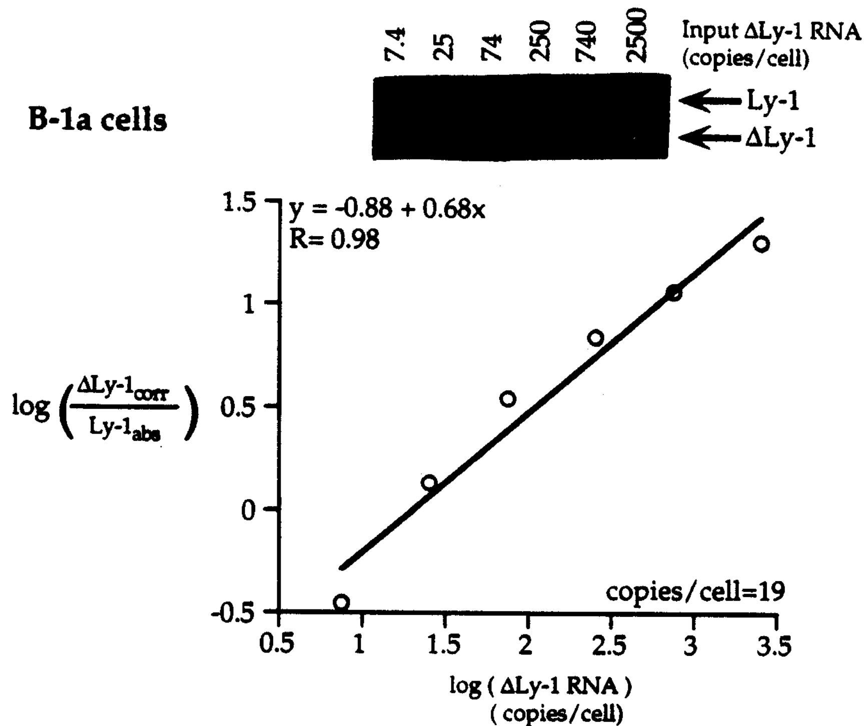
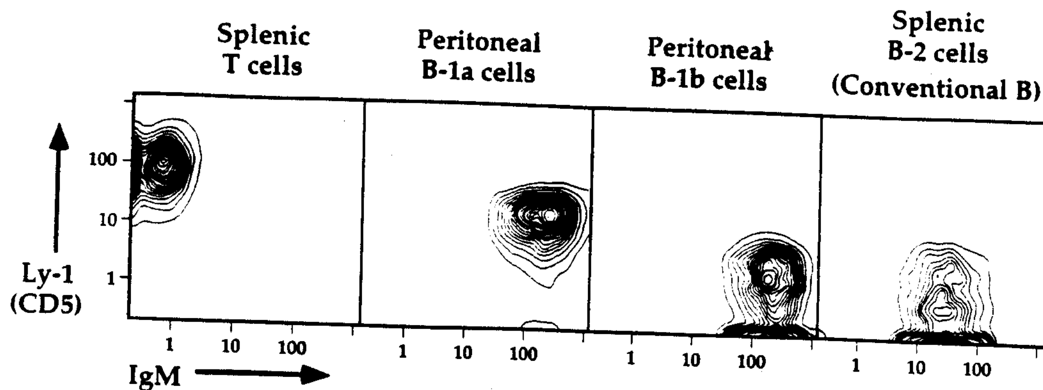


Fig. 59.2. *Quantitation of T and B-1a cells using QC-PCR.* The PCR products were recorded using UVP gel documentation system. The fluorescence of each PCR products was analyzed by NIH Image 1.49 software. The absolute fluorescence (abs) was determined by subtracting the background fluorescence from the measured fluorescence of Ly-1 or ΔLy-1 PCR products. The ΔLy-1 absolute fluorescence was then corrected for size difference ($\Delta Ly\text{-}1_{abs}$•(529/333) = $\Delta Ly\text{-}1_{corr}$). The ratio of $\Delta Ly\text{-}1_{corr}$ and $Ly\text{-}1_{abs}$ was plotted against the known input ΔLy-1 RNA. Endogenous Ly-1 RNA was determined by calculating the amount of input ΔLy-1 RNA at which produced equal amount of Ly-1 and ΔLy-1 PCR product. At the equivalence point, the amount of endogenous Ly-1 RNA was equal to input ΔLy-1 RNA.



Tissue	Cell type	Samples analyzed	Mean RNA (copy/cell)	s.d.
Spleen	T	4	258	55
Peritoneum	B-1a	6	17	6
Peritoneum	B-1b	5	< 2	n.a.
Spleen	B-2	5	< 2	n.a.
Spleen	B-2 + T (0.2%)	3	< 2	n.a.
Spleen	B-2 + T (1%)	3	5	1
Spleen	B-2 + T (5%)	4	16	6

Fig. 59.3. *Reanalyses of the FACS-sorted lymphocyte populations and Ly-1 RNA quantitation for each population as determined by QC-PCR.* Balb/c mice between four to six months old were used for sorting. B-2 and T cells were sorted from spleens. B-1a and B-1b cells were sorted from pooled peritoneal cells. Each population was sorted based on the surface phenotype—T cells: Ly-1$^+$, IgM$^-$; B-2 cells: Ly-1$^-$, IgM^{med}; B-1a cells: Ly-1^{lo}, IgM^{hi}; and B-1b cells: Ly-1$^-$, IgM^{hi}. Each FACS-sorted sample contains 3×10^5 cells. PCR was done at 37 cycles for T cells and 41 cycles for B-1a, B-1b, and B-2 cells.

viremia in the plasma after administration of antiviral compounds [12, 16, 17]. In addition, the induction of a particular gene [13, 18] and changes in cytokine mRNA levels [15, 19, 20] after stimulations can be determined by QC-PCR. We present an example from our studies of murine Ly-1 (CD5) message.

Experiments and results

B-1a cells are a small subset of B cells that express the surface antigen CD5, previously known as a pan T cell marker [21, 22]. They are distinguishable from the majority of B cells in the animal, conventional B (B-2) cells, which lack surface CD5. B-1a cells also have higher IgM and lower IgD surface expression than B-2 cells. Further characterization has identified B-1b cells as a related subset [23]. Both B-1a and B-1b cells share identical surface phenotype except that Ly-1, the murine CD5 homologue, is not found on the surface of B-1b cells. B-1a, B-1b, and B-2 cells can be functionally distinguished from each other by ontogeny, anatomical localization, antibody specificity, self-replenishment, and by the nature of their progenitors (for review, see [24]).

We used QC-PCR to determine the level of Ly-1 message in T and B cells and to address the question whether B-1b cells express Ly-1 message. The experiment is based on the QC-PCR method outlined in Figure 59.1. We constructed a reference template (ΔLy-1) by deleting a portion of Ly-1 cDNA between the two PCR primers. The competitor for Ly-1 RNA (ΔLy-1 RNA) is *in vitro* transcribed from the ΔLy-1 template. The two RNA species yield distinct PCR products of 333 bp (ΔLy-1) or 529 bp (Ly-1).

Each lymphocyte population was sorted using a fluorescence activated cell sorter (FACS) [25]. Total RNA was prepared from each sample. Equal amounts of RNA were aliquoted to a series of tubes containing diluted ΔLy-1 RNA before cDNA synthesis. The cDNA products were then amplified by PCR and subsequently separated by agarose gel electrophoresis. Inspection of the images in Figure 59.2 gives the immediate result that CD8$^+$ thymocytes have more Ly-1 message than B-1a cells, that is, for CD8$^+$ thymocytes, the Ly-1 and ΔLy-1 PCR products are visually comparable at 250 copies of input ΔLy-1 RNA per cell, although for B-1a cells, the PCR products are most similar at 25 copies of input ΔLy-1 RNA per cell. The ratio of ΔLy-1 and Ly-1 PCR products was plotted against the amount of ΔLy-1 RNA added, as shown in Figure 59.2. The amount of endogenous Ly-1 message was determined by calculating the amount of input ΔLy-1 RNA needed to produce equal amount of Ly-1 and ΔLy-1 PCR products. In practice, three or more independent determinations for each cell population were averaged.

FACS reanalyses and mean quantitation for each of the lymphocyte populations are shown in Figure 59.3. In this experiment, B-2 cells were sorted as negative controls. In addition, various amounts of T cells were added back to sorted B-2 cells as controls for T cell contamination. It was determined that B-1a cells express low amounts of Ly-1 message, at about 17 copies per cell. Splenic T cells contain approximately 258 copies of Ly-1 RNA. The Ly-1 RNA quantitation calculated for both B-1b and B-2 cells is less than two copies per cell. This is the limit of sensitivity for the assay. This background level of Ly-1 message in both B-1b and B-2 cells would still be observed even with up to 0.5% T cell contamination. Thus, within the limits set by the contamination control, we determine that B-1b cells do not express Ly-1.

Discussion

QC-PCR is a very sensitive technique for quantitating RNA messages from small numbers of cells. It provides a sensitivity unmatched by Northern hybridization and RNase protection. It provides an internal control for cDNA synthesis and the subsequent PCR amplification by using a reference RNA template that competes with the target template for the same set of PCR primers, and therefore eliminates many of the difficulties encountered when using standard PCR for quantitation.

A couple of points are worth mentioning when using QC-PCR for quantitating from small amount of cells. First, it is important to determine the number of PCR cycles needed. Lowering the amount of target message used in the experiment would require increasing the number of PCR cycles but less competitor templates will be needed. In addition, although the target and reference templates are very well matched in QC-PCR compared with the housekeeping gene approach, the amplification efficiency of one template versus another could become different after high number of cycles due to exhaustion of nucleotides, primers, *Taq* polymerase, or any combination listed above [15, 26]. It is also important to always include negative controls to determine the limits of the assay and how possible contamination could affect the sensitivity. In our study, we sorted B-2 cells as a negative control and evaluated the consequence of low levels of T cell contamination in the sorted populations. Thus, we were able to determine that B-1b cells do not express measurable Ly-1 message, because the quantitated Ly-1 RNA levels for both B-2 and B-1b were equal and less than two copies per cell.

Several researchers have reported generation of recombinant templates for use as internal controls for QC-PCR. One of the recombinant templates consists of a spacer sequence flanked by several sets of PCR binding sites [27]. Another recombinant template is created by linking in tandem short stretches of modified cDNAs from several cytokines [28]. These methods allow for the use of just one competitor construct for quantitation of two or more target genes, thus reducing the amount of handling necessary [29–31]. The precise quantitation, sensitivity, small sample size requirement, and minimal handling should make QC-PCR an invaluable tool for both basic research as well as diagnosis and tracking of disease progression.

Acknowledgment

This work was supported by National Institutes of Health grant AI34762 to LAH and ABK and National Eye Institute grant EY07106 to JT.

References

1. Chelly J, Kaplan JC, Maire P, Gautron S, Kahn A: Transcription of the dystrophin gene in human muscle and non-muscle tissue. *Nature* 333:858–860, 1988
2. Mocharla H, Mocharla R, Hodes ME: Coupled reverse transcription-polymerase chain reaction (RT-PCR) as a sensitive and rapid method for isozyme genotyping. *Gene* 93:271–275, 1990
3. Murphy E, Hieny S, Sher A, O'Garra A:. Detection of in vivo expression of interleukin-10 using a semi-quantitative polymerase chain reaction method in Schistosoma mansoni infected mice. *J Immunol Methods* 162:211–223, 1993
4. Murphy LD, Herzog CE, Rudick JB, Fojo AT, Bates SE: Use of the polymerase chain reaction in the quantitation of mdr-1 gene expression. *Biochemistry* 29:10351–10356, 1990
5. Delidow BC, Peluso JJ, White BA: Quantitative measurement of mRNAs by polymerase chain reaction. *Gene Anal Tech* 6:120–124, 1989
6. Siebert PD, Fukuda M: Induction of cytoskeletal vimentin and actin gene expression by a tumor-promoting phorbol ester in the human leukemic cell line K562. *J Biol Chem* 260:3868–3874, 1985
7. Chen E, Karr RW, Frost JP, Gonwa TA, Ginder GD: Gamma interferon and 5-azacytidine cause transcriptional elevation of class I major histocompatibility complex gene expression in K562 leukemia cells in the absence of differentiation. *Mol Cell Biol* 6:1698–1705, 1986
8. Schmidt EE, Merrill GF: Changes in dihydrofolate reductase (DHFR) mRNA levels can account fully for changes in DHFR synthesis rates during terminal differentiation in a highly amplified myogenic cell line. *Mol Cell Biol* 11:3726–3734, 1991
9. Becker-Andre M, Hahlbrock K: Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). *Nucleic Acids Res* 17:9437–9446, 1989
10. Wang AM, Doyle MV, Mark DF: Quantitation of mRNA by the polymerase chain reaction [published erratum appears in *Proc Natl Acad Sci USA* 87:2865, 1990]. *Proc Natl Acad Sci USA* 86:9717–9721, 1989
11. Gilliland G, Perrin S, Blanchard K, Bunn HF: Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. *Proc Natl Acad Sci USA* 87:2725–2729, 1990
12. Piatak M, Jr, Saag MS, Yang LC, Clark SJ, Kappes JC, Luk KC, Hahn BH, Shaw GM, Lifson JD: High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science* 259:1749–1754, 1993
13. Siebert PD, Larrick JW: Competitive PCR. *Nature* 359:557–558, 1992
14. Xu Z, Dai B, Dhruva B, Singh P: Gastrin gene expression in human colon cancer cells measured by a simple competitive PCR method. *Life Sci* 54:671–678, 1994
15. Kozbor D, Hyjek E, Wiaderkiewicz R, Wang Z, Wang M, Loh E: Competitor mRNA fragments for quantitation of cytokine specific transcripts in cell lysates. *Mol Immunol* 30:1–7, 1993
16. Piatak M, Jr, Luk KC, Williams B, Lifson JD: Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species. *Biotechniques* 14:70–81, 1993
17. Wei X, Ghosh SK, Taylor ME, Johnson VA, Emini EA, Deutsch P, Lifson JD, Bonhoeffer S, Nowak MA, Han BH, Saag MS, Shaw GM: Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* 373:117–122, 1995
18. Vanden Heuvel JP, Clark GC, Thompson CL, Mccoy Z, Miller CR, Lucier GW, Bell DA: CYP1A1 mRNA levels as a human exposure biomarker: use of quantitative polymerase chain reaction to measure CYP1A1 expression in human peripheral blood lymphocytes. *Carcinogenesis* 14:2003–2006, 1993
19. Carding SR, Lu D, Bottomly K: A polymerase chain reaction assay for the detection and quantitation of cytokine gene expression in small numbers of cells. *J Immunol Methods* 151:277–287, 1992
20. Andus T, Targan SR, Deem R, Toyoda H: Measurement of tumor necrosis factor alpha mRNA in small numbers of cells by quantitative polymerase chain reaction. *Reg Immunol* 5:11–17, 1993

21. Lanier LL, Warner NL, Ledbetter JA, Herzenberg LA: Expression of Lyt-1 antigen on certain murine B cell lymphomas. *J Exp Med* 153:998–1003, 1981
22. Hardy RR, Hayakawa K, Haaijman J, Herzenberg LA: B-cell subpopulations identified by two-colour fluorescence analysis. *Nature* 297:590–591, 1982
23. Kantor AB, Stall AM, Adams S, Herzenberg LA, Herzenberg LA: Differential development of progenitor activity for three B-cell lineages. *Proc Natl Acad Sci USA* 89:3320–3324, 1992
24. Kantor AB, Herzenberg LA: Origin of murine B cell lineages. *Annu Rev Immunol* 11:501–538, 1993
25. Parks DR, Herzenberg LA, Herzenberg LA: Flow cytometry and fluorescence activated cell sorting, in *Fundamental Immunology*, 2nd ed, edited by Paul WE, New York, Raven, 1989, pp 781–802
26. Innis MA, Gelfand DH: Optimization of PCRs, in *PCR Protocols: A Guide to Methods and Applications*, edited by Innis MA, Gelfand DH, Sninisky JJ, White TJ, San Diego, Academic Press, 1990, pp 3–12
27. Vanden Heuvel JP, Tyson FL, Bell DA: Construction of recombinant RNA templates for use as internal standards in quantitative RT-PCR. *Biotechniques* 14:395–398, 1993
28. Reiner SL, Zheng S, Corry DB, Locksley RM: Locksley. Constructing polycompetitor cDNAs for quantitative PCR. *J Immunol Methods* 165:37–46, 1993
29. Corry DB, Reiner SL, Linsley PS, Locksley RM: Differential effects of blockade of CD28–B7 on the development of Th1 or Th2 effector cells in experimental leishmaniasis. *J Immunol* 153:4142–4148, 1994
30. Reiner SL, Zheng S, Wang ZE, Stowring L, Locksley RM: Leishmania promastigotes evade interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from CD4+ T cells during initiation of infection. *J Exp Med* 179:447–456, 1994
31. Wang ZE, Zheng S, Corry DB, Dalton DK, Seder RA, Reiner SL, Locksley RM: Interferon gamma-independent effects of interleukin 12 administered during acute or established infection due to Leishmania major. *Proc Natl Acad Sci USA* 91:12932–12936, 1994
32. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987