tration and measured fluorescence of the data shown in Figure 2 was obtained [Bartlett's nonparametric Model II regression analysis (7)] and was represented by the equation: $\text{Log}(\text{DNA}) = -13.50 + 4.25 \text{Log}(\text{Fluorescence})$. This equation can be used to estimate the concentration of DNA in a solution from fluorescence measured, following the protocol outlined above.

Although estimating DNA concentrations might look trivial, the outcome of several experiments depends on the accurate determinations of DNA in the samples or stock DNA preparations. PCRs can be inhibited by excessive DNA concentrations, and no amplification product will be obtained if DNA is below the amplification threshold. Concentrations of genomic DNA are tricky to estimate because of the viscosity of the concentrated solutions and the unevenness of long DNA in more diluted solutions. This easy and accurate protocol will facilitate the estimation of DNA concentrations. In our laboratory, we perform serial dilutions of genomic DNAs over several orders of magnitude to obtain an accurate estimation of DNA concentrations in stock solutions. The use of a 96-well plate or a multi-tube approach facilitates this strategy, and the iQ iCycler optical thermal cycler can easily be used for this purpose. The protocol outlined in this study avoids the need to purchase costly fluorescence plate readers, learn how to use an extra apparatus, and simultaneously saves precious bench space.

REFERENCES

1. **Morrison, T.B., J.J. Weis, and C.T. Wittwer.** 1998. Quantitation of low copy transcripts by continuous SYBR Green I monitoring during amplification. *BioTechniques* 24:954-962.
  2. **Akey, J.M., D. Sosnoski, E. Parra, S. Dios, K. Hiester, B. Su, C. Bonilla, L. Jin, et al.** 2001. Melting curve analysis of SNPs (Mc-SNP): a gel-free and inexpensive approach for SNP genotyping. *BioTechniques* 30:358-367.
  3. **Ririe, K.M., R.P. Rasmussen, and C.T. Wittwer.** 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal. Biochem.* 245:145-160.
  4. **Gonzalez, J. M. and C. Saiz-Jimenez.** 2002. A fluorimetric method for the estimation of G+C mol% content in microorganisms by thermal denaturation temperature. *Environ. Microbiol.* 4:770-773.
  5. **Molecular Probes.** 1996. SYBR Green I nucleic acid gel stain. *Molecular Probes Application Sheet MP-7567.*
  6. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. *Molecular Cloning: A Laboratory Manual.* CSH Laboratory Press, Cold Spring Harbor, NY.
  7. **Sokal, R.R. and F.J. Rohlf.** 1981. *Biometry*, 2nd ed. W.H. Freeman and Co., New York, NY.
- We acknowledge support from the Spanish Ministry of Science and Technology through the Ramón y Cajal Program and projects REN2002-00041 and BTE2002-04492-C02-01. Address correspondence to Dr. Juan M. Gonzalez, IRNAS-CSIC, Apdo 1052, 41080, Sevilla, Spain. e-mail: jmgrau@irnase.csic.es*

Received 19 December 2002; accepted 24 January 2003.

**Juan M. Gonzalez and
Cesareo Saiz-Jimenez**
*Instituto de Recursos Naturales
y Agrobiología
CSIC, Sevilla, Spain*

Modified 3'-End Amplification PCR for Gene Expression Analysis in Single Cells

BioTechniques 34:712-716 (April 2003)

For years, gene expression has been widely studied in most fields of biology. It has been used to address molecular mechanisms controlling basic cell functions and their alterations, including cell division, cell differentiation, apoptosis, and various infectious processes. In the vast majority of such studies, carried out by conventional techniques such as Northern blotting or RT-PCR, only the mean gene expression within large populations of cells was analyzed, and cell-to-cell variability was not taken into account. Remarkably, several independent studies using in situ detection techniques have recently pointed to the existence of important intercellular variability in gene expression, both in prokaryotic and mammalian cells. Altogether, the data strongly argue for a stochastic-based process of gene expression (1-4). To further our understanding of cell physiology, there is an increasing need to evaluate intercellular variability of gene expression in a large number of cell types and physiological situations. To this end, several techniques have recently been developed, each with various advantages and limitations (5).

In the course of our work, we used the 3'-end amplification PCR (TPEA-PCR) procedure, which has proven to be an efficient tool for studying gene expression in various mammalian cell lines at the single-cell level. Although this technique is not quantitative, it allows virtually all the mRNAs sequences present in a single cell to be amplified, including those present at low copy number (6). This RT-PCR relies on two successive amplifications. First, cell lysate-containing mRNAs are reverse-transcribed, followed by a non-specific amplification, using primers designed to amplify all polyadenylated mRNAs. Second, there is a gene-specific amplification of up to 40 aliquots of the first amplification product. Thus, it is theoretically possible to detect up

Benchmarks

to 40 different transcripts (20 in duplicates) in a single cell and to follow gene expression patterns and their variations from cell to cell.

However, contamination remains critical, especially when the quantity of RNA in the starting material is limited, as is always the case with single-cell RT-PCR. Decreasing the number of manipulations carried out during the procedure will reduce the risk of contamination. Here we propose such a modification of the TPEA technique (schematically shown in Figure 1) that maintains the quality of the result. We reduced the successive substeps originally required for the nonspecific amplification from seven to four. Specifically, we have combined the two second-strand synthesis substeps with a round of 15 nonspecific PCR cycles. This simplification largely reduces the contamination risk, results in a significant gain of time, and becomes particularly convenient when a large number of cells are to be screened concomitantly. For example, the amplification time required for 30 single cells is 1.5 h shorter than that required for the classical TPEA technique (8 h including cell harvest and lysis).

In our experiments, murine neuroblastoma cells (clone N1E-115) were cultured in DMEM with 4.5 g/L glucose (Invitrogen, Carlsbad, CA, USA) supplemented with 10% decompartmented FBS and 10 mL/L penicillin-streptavidin (Invitrogen).

All the reverse transcription- and PCR-based reactions were carried out in a GeneAmp® PCR 9700 apparatus (Applied Biosystems, Foster City, CA, USA). Reverse transcription primer (5'-CTCTCAAGGATCTTACCGCTTTT-TTTTTTTTTTTTTT-3'), second-strand primer (5'-CTGCATCTATCTAATGCTCCNNNNNCGAGA-3', where N represents C, G, T, or A), and 3'-heel primer (5'-CTCTCAAGGATCTTACCGC-3') were those described by Dixon et al. (6).

After cell harvest and serial dilutions in Terasaki plates, single cells (1 µL) were identified under a light microscope and lysed in situ in a final volume of 7 µL containing 1× first-strand buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂; Invitrogen), 5 mM Nonidet P-40™ (Sigma, St. Louis, MO, USA), 1.5 U RNase Inhibitor RNase-

OUT™ (Invitrogen). After 5 min on ice, nuclei were removed by centrifugation (8000× g for 5 min at 4°C). Each supernatant was then transferred to a new tube and reverse transcribed (first strand) in a final volume of 10 µL containing 1× first-strand buffer, 200 U Moloney murine leukemia virus reverse transcriptase (Invitrogen), and 0.5 ng reverse transcription primer for 60 min at 37°C.

cDNA second-strand synthesis, primer extension, and 15 rounds of PCR were combined as a single step instead of being carried out separately (Figure 1). The 10-µL reverse transcription volume was added to a 10.4-µL PCR mixture and was subjected to 50°C for 15 min (incubation), 72°C for

10 min (primer extension), 92°C for 5 min (denaturation), 15 PCR cycles (2.5 min at 92°C, 60°C for 1.5 min, and 72°C for 1.5 min), and 72°C for 10 min. Final concentrations of this 20.4-µL reaction volume were 4.5 mM MgCl₂, 67 mM Tris-HCl (pH 8.3), 0.5 mM dNTPs (Amersham Biosciences UK, Buckinghamshire, UK), 1 ng second-strand primer, 0.4 ng 3'-heel primer, and 0.35 U AmpliTaq® DNA polymerase (Applied Biosystems).

The 20.4-µL RT-PCR volume was then increased to 30.4 µL without changing reagent concentrations, except for the addition of 125 ng second-strand primer and 50 ng 3'-heel primer, and subjected to denaturation at 92°C for 5 min, 25 additional PCR cycles (as

Figure 1. Original (A) and modified (B) TPEA-PCR protocols.

Figure 2. Agarose gel electrophoresis of β -actin (A), GAPDH (B), and insulin receptor (Ins-R; C) cDNAs. The modified TPEA-PCR protocol was used to detect the expression of the three genes in 11 single NE1-115 cells (lanes a–k). Lane l, molecular weight marker (100-bp DNA ladder; New England Biolabs). Lane m, negative control (no reverse transcriptase). Lane n, negative control (no cell). Primers were the following: β -actin (209 bp; GenBank® accession no. V01217) 5'-CATCCATGCCCT-GAGTCC-3' (forward) and 5'-ACACCTCAAACCACTCCAG-3' (reverse); GAPDH (408 bp; accession no. X02231) 5'-GACCCCTTCATTGACCTCAACTAC-3' (forward) and 5'-GATGC-CAAAGTTGTCATGGATGAC-3' (reverse); and insulin receptor (369 bp; accession no. NM 010568) 5'-TCACAAAGATGGCGTATT-3' (forward) and 5'-GACTGGAGCAAACACAAC-3' (reverse).

described above), and a final elongation at 72°C for 10 min.

Additionally, we improved the yield of the second gene-specific PCR by using only 3 μ L of the nonspecific amplification product, instead of 5 μ L. The potential number of gene-specific PCRs is thereby increased from 40 to 50. Thus, the 30.4- μ L final reaction volume was diluted to a 150- μ L final volume in (10 mM Tris-HCl, 0.1 mM EDTA), and 3 μ L of this solution were used for the second PCR using gene-specific primers. Each sample was subjected to PCR in a 50- μ L final volume reaction containing at final concentrations: 1 $\times$ PCR Buffer II (100 mM Tris-HCl, 500 mM KCl; Applied Biosystems), 3.5 mM MgCl₂ (Applied Biosystems), 12.5% sucrose, 12 mM β -mercaptoethanol, 0.5 mM dNTPs, 0.6 U AmpliTaq, and primers at 100 ng/reaction. Cresol red was found to be unnecessary and was therefore omitted. Annealing temperatures were adjusted when necessary, but otherwise PCR conditions were those used by Dixon et al. (6).

The modified TPEA protocol gave sensitive and reproducible results. In

Figure 2, we show the results we obtained using three different sets of primers specific for β -actin (A), GAPDH (B), and the insulin receptor (C) genes. Importantly, no signal was detected when reverse transcriptase was omitted, demonstrating the absence of genomic DNA amplification. This result was strengthened by the fact that the GAPDH primers used for this amplification were intron spanning.

As expected, the two constitutive genes (β -actin and GAPDH) were found to be expressed in all the single cells. In contrast, expression of the insulin receptor gene, which is not constitutively produced in N1-E115 neuroblastoma cells, was found to be variable and was detected in four of 11 cells.

As an additional control, we have also divided cDNA pools in two after the reverse transcription step and compared the signals obtained after the full TPEA procedure was performed with β -actin primers. No bias in the amplification of transcripts was detected (data not shown). Altogether, these modifications result in a faster, more efficient, and more reliable TPEA-PCR technique.

REFERENCES

1. Elowitz, M.B., A.J. Levine, E.D. Siggia, and P.S. Swain. 2002. Stochastic gene expression in a single cell. *Science* 297:1183-1186.
2. De Krom, M., M. van de Corput, M. von Lindern, F. Grosveld, and J. Strouboulis. 2002. Stochastic patterns in globin gene expression are established prior to transcriptional activation and are clonally inherited. *Mol. Cell* 9:1319-1326.
3. Hume, D.A. 2000. Probability in transcriptional regulation and its implications for leukocyte differentiation and inducible gene expression. *Blood* 96:2323-2328.
4. Kupiec, J.J. 1996. A chance-selection model for cell differentiation. *Cell Death Differ.* 3:385-390.
5. Todd, R. and D.H. Margolin. 2002. Challenges of single-cell diagnostics: analysis of gene expression. *Trends Mol. Med.* 8:254-257.
6. Dixon, A.K., P.J. Richardson, K. Lee, N.P. Carter, and T.C. Freeman. 1998. Expression profiling of single cells using 3 prime end amplification (TPEA) PCR. *Nucleic Acids Res.* 26:4426-4431.

We are grateful to Anne Tyvaert for technical assistance. We thank Drs. Sitbon, Taylor (Montpellier), and Tardieux (Paris) for support and critical reading of this manuscript. We are grateful to Dr. Sonigo for constant support and helpful advice. Address correspondence to Dr. Jean-Jacques Kupiec, Equipe de Génétique des virus, Institut Cochin, INSERM U567, CNRS UMR 8104, 22 rue Méchain, 75014 Paris, France. e-mail: kupiec@cochin.inserm.fr

Received 23 December 2002; accepted 7 February 2003.

Thomas Heams and Jean-Jacques Kupiec
Institut Cochin
Equipe de Génétique des virus
INSERM U567 and
CNRS UMR 8104
Paris, France