Representation is faithfully preserved in global cDNA amplified exponentially from sub-picogram quantities of mRNA

Norman N. Iscove[1,3]*, Mary Barbara[1,3], Marie Gu[1,3], Meredith Gibson[2], Carolyn Modi[2], and Neil Winegarden[2]

Published online: 12 August 2002; corrected 19 August 2002 (details online); doi:10.1038/nbt729

© 2002 Nature Publishing Group http://www.nature.com/naturebiotechnology

Analysis of transcript representation on gene microarrays requires microgram amounts of total RNA or DNA. Without amplification, such amounts are obtainable only from millions of cells. However, it may be desirable to determine transcript representation in few or even single cells in aspiration biopsies, rare population subsets isolated by cell sorting or laser capture, or micromanipulated single cells. Nucleic-acid amplification methods could be used in these cases, but it is difficult to amplify different transcripts in a sample without distorting quantitative relationships between them. Linear isothermal RNA amplification has been used to amplify as little as 10 ng of total cellular RNA, corresponding to the amount obtainable from thousands of cells, while still preserving the original abundance relationships[1,2]. However, the available procedures require multiple steps, are labor intensive and time consuming[3], and have not been shown to preserve abundance information from smaller starting amounts. Exponential amplification, on the other hand, is a relatively simple technology, but is generally considered to bias abundance relationships unacceptably[2–5]. These constraints have placed beyond current reach the secure and routine application of microarray analysis to single or small numbers of cells. Here we describe results obtained with a rapid and highly optimized global reverse transcription–PCR (RT-PCR) procedure. Contrary to prevalent expectations, the exponential approach preserves abundance relationships through amplification as high as 3×10^{11}-fold. Further, it reduces by a million-fold the input amount of RNA needed for microarray analysis, and yields reproducible results from the picogram range of total RNA obtainable from single cells.

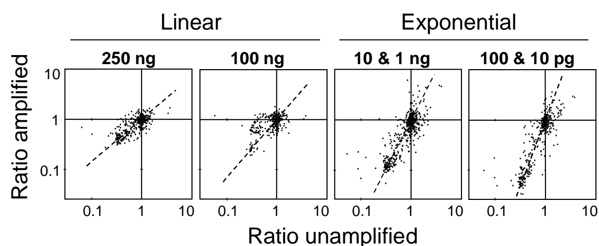
Exponential amplification is believed to degrade abundance relationships because cDNAs of differing lengths and composition would be amplified with differing efficiencies[2–5]. The particular method for global amplification of mRNA used in this study was designed to minimize such bias[6–8]. The procedure involves reverse transcription of a first cDNA strand primed by oligo(dT), addition of an oligo(dA) tail with terminal transferase, and subsequent exponential amplification using a single oligo(dT)-containing primer. A major design consideration was to maximize the efficiency of capture of individual transcripts, even those present at low abundance in small samples. A procedure was therefore developed that did not depend on nucleic acid extractions, ligations, full-length transcription, or template switching, each of which would have introduced probabilistic effects and potential bias. The most important design choice was to limit the extent of reverse transcription to only a few hundred bases of extreme 3′ sequence by limiting deoxynucleotide concentrations and the time of the reaction. These conditions were intended to provide a more uniform likelihood of sampling individual mRNA transcripts, and a more uniform amplification efficiency across all cycles. The procedure has been applied extensively to small samples and frequently to single micromanipulated cells[8–19]. Although this experience provided evidence of preservation of quantitative information in amplified samples, only small numbers of probes were tested, and it remained to be established quantitatively whether abundance relationships were preserved broadly enough to make exponentially amplified cDNA suitable for analysis on cDNA microarrays.

Our experiments were directed at determining the extent to which cDNA amplified by global RT-PCR retained the abundance relationships characteristic of the original mRNA. We chose to examine differences in transcript abundance between two RNA samples of differing cellular origin: a single preparation of total RNA from HeLa cells (a human cervical epithelial cell line), and a commercial Universal Human Reference RNA pooled from ten cell lines representing distinct cell lineages (cervical, mammary, hepatic, and embryonal carcinomas; histiocytic, B- and T-cell leukemias; and melanoma, glioblastoma, and liposarcoma). We expected that large numbers of lineage-specific transcripts would be represented more abundantly in the Universal Reference sample than in the HeLa cell sample. To measure abundance differences, we used microarrays of 1,718 distinct human expressed sequence tag (EST) probes, each spotted in duplicate, and many of which contained extreme 3′ transcript sequence.

To test the fidelity of target amplification, we determined the ratios of HeLa to Universal Reference hybridization on individual probes obtained with amplified targets ("test ratios"), and examined how these correlated with the corresponding ratios obtained with unamplified targets ("true ratios"). RNA was amplified either by a single round of linear isothermal RNA amplification, or by exponential RT-PCR, to obtain a direct comparison of fidelities achieved by each method. Each individual array was hybridized with a mixture of HeLa and Universal Reference cDNA targets prepared by the same method and differentially labeled with Cy3 and Cy5. Cy3 and Cy5 intensity ratios were then determined for each spotted probe. For both true and test ratio sets, a major cluster of normalized HeLa/Universal ratios near 1, and a minor cluster of ratios <0.5, were readily apparent. We plotted test ratios against true HeLa/Universal ratios obtained for linear and exponential methods applied to differing starting amounts of total RNA (Fig. 1). Test ratios obtained for targets prepared by linear RNA amplification from 250 ng or 100 ng of RNA showed the expected positive correlation with true ratios. However, we obtained more striking results with exponentially amplified targets, for which the extent of correlation with true ratios was stronger than that obtained with linear amplification, and for which correlation did not decay even with a final fold amplification as high as 3×10^{11} from as little as 10 pg of RNA. The slope of the relationship between test and true ratios was also considerably steeper with exponentially amplified targets, so that ratios <1 determined with amplified targets were smaller than those obtained with unamplified targets. The steepness of the slope is believed to be related to the double-stranded nature of the targets generated in the exponential protocol and the resulting competition for hybridization between immobilized probe and opposite strands in solution.

[1]Department of Cell and Molecular Biology and [2]Microarray Centre, The Ontario Cancer Institute, 610 University Avenue, Toronto, ON, Canada M5G 2M9. [3]Department of Medical Biophysics, University of Toronto, Toronto, ON, Canada M5G 2M9. *Corresponding author (iscove@uhnres.utoronto.ca).

© 2002 Nature Publishing Group http://www.nature.com/naturebiotechnology



Amplification	225×	725×	$3 \times 10^8/10^9$	$3 \times 10^{10}/10^{11}$
Items	401	299	549	481
Slope	0.83	1.15	1.88	2.50
R^2	0.54	0.31	0.66	0.74

Figure 1. Scatter plots showing the relationship between normalized HeLa/Universal hybridization ratios obtained with amplified and unamplified targets. Each dot represents the average of four ratios from duplicate non-blank spots on duplicate arrays, where all included spots had 4/4 intensity values above filter threshold. Starting amounts of total RNA are indicated above each plot. Ratios were averaged from two arrays for 250 ng; two arrays for 100 ng; one array for 10 ng with one array for 1 ng; and one for 100 pg with one for 10 pg. Regression lines were forced through the origin. Slopes and Pearson correlation coefficients were computed from the ratio logarithms. Beneath each panel are given the total amplification factor from starting mRNA amount through to labeled target, the number of plotted points, the slope, and the Pearson R^2 value. Amounts of amplification product were estimated by densitometry on agarose–ethidium bromide gels. Of total RNA, mRNA was assumed to comprise 2%.

Together, the results show that abundance relationships are preserved in our protocol for exponential amplification at least as well as they are during a single round of linear RNA amplification, and over a much greater amplification range.

The main practical application of microarray analysis is the identification of transcripts whose abundance differs between samples. We assessed utility directly by testing whether amplified targets would identify the same set of differentially expressed transcripts recognizable with unamplified targets. As most outlying HeLa/Universal hybridization ratios in both unamplified and amplified datasets were <1 (Fig. 1), we confined our attention to outlier ratios ≤0.5. Individual probes from duplicate hybridizations with unamplified targets were accepted as "true outliers" if four of four spots had ratios ≤0.5. The corresponding probes from duplicate hybridizations with amplified targets were similarly accepted as "matches" if four of four spots had ratios ≤0.5. The results obtained for linearly and exponentially amplified target preparations are summarized in Table 1. On average, linearly amplified targets identified 34% ((28 + 42)/208) of true outliers. In contrast, exponentially amplified targets identified 75% of the true outliers. Ratios of true outliers were overestimated (false negatives) in only 5% of instances for exponential amplification, but in 29% of instances for linear amplification.

The proportions of true outlier probes that failed to be illuminated above background for exponentially or linearly amplified targets were 20% and 35%, respectively. Because targets amplified exponentially by our protocol contain only 3′ transcript termini, they would not be expected to hybridize to probes that do not contain 3′ termini. About one-third of the probes on our arrays contain polyadenylation signals, but further characterization of their positioning remains to be done. Linearly amplified targets, in contrast, would not be expected to have an equivalent bias toward 3′ content, yet they illuminated even fewer probes than did exponentially amplified targets. The basis of this observation has not been identified.

In addition to true outliers, exponentially amplified targets also identified large numbers of probes with test ratios <0.5 (false positives) and true ratios >0.5. However, many (55%) of these probes actually had true mean ratios ≤0.6. Some of these instances apparently reflected the exaggeration of low ratio values by exponential amplification evident in Figure 1. With the foreknowledge that the slope of the relationship between the logarithms of test and true ratios is near 2 (Fig. 1), corresponding true ratios can be approximated from observed test ratios using the relationship:

$$\text{Estimated true ratio} = e^{(\ln(\text{Test ratio}))/2}$$

Application of this transform indeed eliminated over half of the false positives without substantial impact on the yield of true matches (data not shown).

Each of four microarrays hybridized with exponentially amplified targets identified essentially the same set of 74 probes underrepresented in HeLa cell RNA relative to Universal Reference RNA (Table 1, legend). Of these, 22 are listed to illustrate the successful detection of transcripts likely to be expressed specifically in lineages included in the Universal Reference pool (Table 2). Collectively, these results show that the anticipated superiority of linear amplification to exponential amplification failed to materialize in a head-to-head comparison. Exponentially amplified targets yielded tighter correlations between test- and true-probe ratios and more accurate identification of true outliers. Although we performed only a single round of linear amplification and others have reported improved precision after a second round, our results for exponential amplification are at least comparable to the best results reported by workers expert in the linear method starting from amounts of 10 ng of RNA or more[1]. The two-round linear protocol, spanning in total a week or more, involves repeated steps of reverse transcription, DNA polymerization, DNA concentration and isolation, RNA polymerization

Table 1. Sensitivity and accuracy of identification of outlying hybridization ratios using amplified targets

	Of 104 probes with true ratio ≤0.5, number hybridized by amplified targets at indicated test ratios			Of probes with true ratio >0.5, number hybridized with low test ratio by amplified targets
Target	**Matches (≤0.5)**	**False negatives (>0.5)**	**No hybridization**	**False positives (≤0.5)**
Linear				
250 ng	28	49 (19)[a]	27	5
100 ng	42	12	50	16
Exponential				
10 & 1 ng	76[c]	8 (5)[a]	20	50[b]
100 & 10 pg[d]	80[c]	2	22	57[b]

For each probe, ratios were averaged from four duplicate spots on duplicate arrays paired as in Figure 1. True outliers had HeLa/Universal ratios ≤0.5 on 4/4 probes with unamplified targets, with all intensities above filter threshold. Of probes with ratios ≤0.5 on 4/4 spots with amplified targets, matches identified true outliers, whereas those that were not concordant represented false positives. In each row, matches, false negatives, and no hybridizations summed to 104, the number of true outliers.
[a]Boundary cases: 19/49 and 5/8, which had test mean ratios ≤0.5 but were excluded from the matches category because of nonunanimity.
[b]Of these false positives, 55% of these were boundary cases with true mean ratios ≤0.6 with unamplified probes.
[c]These match values have 74 items in common, including entities with likely lineage specificity corresponding to lineages represented in the Universal Reference RNA.
[d]Similar results were obtained when the 10 pg dataset was analyzed alone.

© 2002 Nature Publishing Group http://www.nature.com/naturebiotechnology

Table 2. Lineage-specific transcripts identified as underrepresented in HeLa cell RNA with exponentially amplified targets

Macrophage colony-stimulating factor I receptor precursor (c-fms)
Vascular endothelial growth factor receptor 2 precursor
Eosinophil granule major basic protein precursor (mbp)
Pleckstrin (platelet p47 protein)
Proto-oncogene c-crk
Endothelin-1 precursor (et-1)
Elastin precursor (tropoelastin)
Prothrombin precursor (coagulation factor II)
α-2-hs-glycoprotein precursor (fetuin) (α-2-z-globulin)
Cathepsin S precursor
Thyroxine-binding globulin precursor
Osteonectin
Erythrocyte adducin α-subunit
Melanoma-associated antigen 10 (mage-10 antigen)
Complement receptor type 1 precursor (c3b/c4b receptor) (cd35 antigen)
Colorectal mutant cancer protein (mcc protein)
Immunoglobulin J chain
Igλ chain C regions
α-1-fetoprotein precursor
Leukocyte adhesion glycoprotein LFA-1 α-chain precursor (integrin α-l)
Blood group rh(ce) polypeptide
Macrophage mannose receptor precursor

and isolation, and cDNA labeling. In contrast, the global RT-PCR protocol, applicable to as little as 10 pg of RNA or a single cell, is in essence a one-tube procedure that can yield micrograms of aminoallyl-derivatized cDNA in the course of a single day. Moreover, the amplified cDNA is a permanent resource that can be reamplified as needed, apparently without compromising abundance relationships. With due regard for the principal limitation of the method—the requirement that probes include sequence at the 3′ transcript termini—the exponential methodology described here has compelling advantages, including its ability to access samples as small as a single cell.

Experimental protocol

RNA isolation. Total RNA was isolated from cultured HeLa cells using an RNeasy Mini Kit (Qiagen, Valencia, CA). Concentration was determined by spectrophotometer (A_{260}) and integrity confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Universal Reference RNA (Stratagene, La Jolla, CA) precipitate in ethanol was pelleted, washed in 70% (vol/vol) ethanol, and dissolved in RNase-free water at 2 µg/µl.

Preparation of unamplified cDNA target. RNA (10 µg) was used for each 40 µl labeling reaction. The reaction buffer contained 1× SuperScript II First Strand buffer (Invitrogen, Carlsbad, CA), 150 pmol primer (5′-T_{20}VN, Cortec, Kingston, ON, Canada), 0.5 mM each of dATP, dGTP, and dTTP (Amersham Biosciences, Piscataway, NJ), 0.05 mM dCTP (Amersham), 0.025 mM of either Cy3 or Cy5 dCTP (Roche Diagnostics Canada, Laval, QC, Canada), and 0.01 mM dithiothreitol (DTT; Invitrogen). The labeling reaction was heated to 65°C for 2 min and then cooled to 42°C. SuperScript II Reverse Transcriptase (2 µl; Invitrogen) was added. After labeling for 2 h, the tubes were centrifuged and placed on ice. EDTA (4 µl of 50 mM, pH 8.0) and 2 µl of 10 M NaOH were added to stop the reaction. RNA was hydrolyzed at 65°C for 20 min. The pH was then neutralized by addition of 5 M acetic acid, and labeled cDNA was purified on PCR cleanup columns (Amicon/Millipore, Bedford, MA).

RNA amplification. For preparation of linearly amplified cDNA target, a single round of RNA amplification was carried out as described previously[1] with minor optimizations. For exponential amplification, the originally described method for global RT-PCR[6,7] was first systematically reconstructed to address deficiencies that could affect fidelity and sensitivity, including amplification of primer concatemers or of sequences lacking polyadenylation signals originating from mispriming on either RNA or genomic DNA templates. This procedure was modified to be more robust and inclusive when applied to single cells, and to generate libraries from single cells in which 72% of clones (vs. <30% before modification) contained polyadenylation signals (data not shown).

Each individual RNA sample was amplified through 65 cycles of PCR, followed by an additional 25 cycles for incorporation of aminoallyl-dUTP. In detail, RNA in 0.5 µl H_2O was added to 4 µl first strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3mM $MgCl_2$, 0.5% (vol/vol) NP-40, 1 mM DTT, 100 µg/ml acetylated BSA, 2.9 µl/100 µl RNAguard (Amersham Biosciences), 0.1 µl/100µl Prime RNase Inhibitor (Eppendorf - 5 Prime, Boulder, CO), 10 µM fresh dNTPs) in a 200 µl PCR tube. After heating to 65°C for 90 s, the tube was cooled to 50°C and reverse transcription was initiated by addition of 0.5 µl (100 units) SuperScript II (Invitrogen) and 0.2 µl SR-T24 primer (5′-GTTAACTCGAGAATTCT_{24}; ref. 14) to a reaction concentration of 0.00245 absorbance units/ml (A_{260}, 3.5 nM). After 15 min, the reaction was terminated by heating to 70°C for 10 min. RNAse H (1.0 µl; Amersham/Pharmacia) and 0.7 µl $MgCl_2$ (75 mM, combined final concentration, 9.4 mM Mg^{2+}) were added and RNA was digested at 37°C for 15 min. The cDNA strands were tailed by addition of 6.5 µl 2× tailing buffer (Roche/Boehringer Mannheim, Mannheim, Germany; final reaction concentrations 25 mM Tris-HCl, 200 mM potassium cacodylate, pH 6.6, 1.5 mM $CoCl_2$) containing dATP to a final reaction concentration of 750 µM and 0.5 µl (25 units) terminal deoxynucleotidyl transferase (Roche/Boehringer Mannheim), and incubated at 37°C for 15 min. Tailing was stopped by heating to 65°C for 10 min. Reaction product (4 µl) was added to each of three tubes containing 15 µl polymerase buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 µg/ml BSA, and 0.05% (vol/vol) Triton X-100), 0.875 mM dNTPs (7.5 absorbance units/ml; A_{260}), and 11 µM SR-T24 primer. The resulting mixture contained totals of 13 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, and residual 0.3 mM $CoCl_2$. To generate the second cDNA strands, the mixture was overlaid with mineral oil and 2 units Taq and 0.05 units Pfu (Stratagene) polymerases were added at 94°C. Primer was annealed at 50°C for 2 min followed by 2 min of extension at 72°C. After amplification through 30 additional cycles (15 s at 94°C; 30 s at 60°C; 2 min at 72°C), 1 µl from each of the three tubes was pooled and 0.2 µl added to 18 µl of polymerase buffer containing 0.2 mM dNTPs and 2.5 µM primer at 1.8 absorbance units/ml; A_{260}. The mixture was overlaid with mineral oil, Taq and Pfu polymerases were added at 94°C, and an additional 35 cycles of amplification were carried out. Amplified stock cDNA was stored at –20°C. Starting from 10 pg of total RNA, or 0.2 pg of mRNA, the entire procedure yields 6 µg of stock cDNA, a 3×10^7-fold amplification over the original amount of mRNA.

Generation of dye-coupled cDNA. Diluted amplified stock cDNA (1:100, 1 µl) was added to 98 µl polymerase buffer containing 0.5 absorbance units/ml; A_{260}, 0.7 µM SR-T24 primer, 0.1 mM dTTP, and 0.2 mM each of dCTP, dGTP, dATP and aminoallyl-dUTP (Sigma-Aldrich, St. Louis, MO). Taq polymerase (2 units) was added at 94°C, and the mixture was amplified through 25 cycles (15 s at 94°C; 30 s at 60°C; 1 min at 72°C). This step entails a further 10^4-fold amplification. Aminoallyl cDNA (typically 5 µg) was purified on a Microcon-30 column (Millipore) according to directions, and concentrated to 2–3 µl by centrifugation under vacuum, and 1 µl was added to 5 µl of 0.1 M $NaHCO_3$, pH 9.0. The contents of one vial of Cy3 or Cy5 monofunctional reactive dye (Amersham/Pharmacia) was dissolved in 45 µl dimethyl sulfoxide. Aminoallyl cDNA (5 µl) was mixed with 5 µl of dye and the tube was wrapped in foil to exclude light and incubated 30 min at room temperature. Labeled cDNA was isolated using a High Pure PCR purification kit (Boehringer Mannheim) according to directions, and the eluate was concentrated to 5–7 µl by vacuum centrifugation. Labeling efficiency was measured in some samples by absorbance at 260 nm and 550 nm for Cy3 or 650 nm for Cy5. Arrays were hybridized with ~1 µg each of HeLa and Universal Reference cDNA.

For analysis of targets on microarrays, hybridization buffer (DIG Easy Hyb, Roche Diagnostics Canada) containing 50 µg each of yeast tRNA (Invitrogen) and calf thymus DNA (Sigma-Aldrich) per 100 µl was added to concentrated Cy3 and Cy5 labeled cDNA to a total volume of 50 µl, heated to 65°C for 2 min, and pipetted onto a 24 × 30 mm coverslip. A microarray slide was lowered onto the coverslip, inverted, placed in a closed, water-containing plastic hybridization chamber, and incubated on a level surface for 16 h at 42°C in a covered water bath. The coverslip was removed by immersion of the array in 1× SSC. The array was washed five times for 5 min at room temperature in 0.1× SSC/0.1% (vol/vol) SDS with agitation, rinsed 3 times in 0.1× SSC, dried by centrifugation, and scanned with minimum delay.

Microarray fabrication and analysis. Microarrays were printed on Corning CMT-GAPSII slides (Corning Life Sciences, Acton, MA) using a Virtek ChipWriter (Virtek Vision, Waterloo, ON, Canada). They consisted of 1,718 distinct PCR-amplified human cDNAs prepared from IMAGE EST clones obtained from Incyte Genomics (St. Louis, MO). Each was spotted in duplicate to give a total of 3,436 spots. In addition, 148 blank spots consisted of either SSC or PCR buffer. Hybridized arrays were scanned on a ScanArray 4000XL (Packard BioChip Technologies, Billerica, MA). The Cy3 and Cy5 channels were balanced by eye with the ScanArray Software to ensure a similar dynamic range for the two channels. Final scans were taken at 10 μm resolution, and images for each channel were saved as separate 16-bit TIFF files. These were imported into GenePix (Axon Instruments, Union City, CA) for quantitation.

For analysis, intensity thresholds were set to exclude 98% of blank spots. Using the Cy3 or Cy5 dataset that yielded the greater number of spots above cutoff, this filter excluded 65% of spotted EST probes on average from further analysis. Raw intensity ratios (HeLa/Universal) were computed for the filtered data and normalized by dividing by the modal ratio.

Acknowledgments
We thank D. Nallainathan for technical assistance, P. Macgregor and M. Albert for their assistance with the linear amplification protocols, C. Holness and colleagues at SyStemix Inc. for DNA sequencing, and P. Subarsky for helpful discussion and advice. This work was supported by an operating grant to N.N.I. from the National Cancer Institute of Canada, and to N.W. from the Ontario Research and Development Challenge Fund.

Competing interests statement
The authors declare that they have no competing financial interests.

Received 28 March 2002; accepted 30 May 2002

© 2002 Nature Publishing Group http://www.nature.com/naturebiotechnology

1. Wang, E., Miller, L.D., Ohnmacht, G.A., Liu, E.T. & Marincola, F.M. High-fidelity mRNA amplification for gene profiling. *Nat. Biotechnol.* **18**, 457–459 (2000).
2. Baugh, L.R., Hill, A.A., Brown, E.L. & Hunter, C.P. Quantitative analysis of mRNA amplification by *in vitro* transcription. *Nucleic Acids Res.* **29**, E29/1–9 (2001).
3. Phillips, J.K. & Lipski, J. Single-cell RT-PCR as a tool to study gene expression in central and peripheral autonomic neurones. *Auton. Neurosci.* **86**, 1–12 (2000).
4. Freeman, T.C., Lee, K. & Richardson, P.J. Analysis of gene expression in single cells. *Curr. Opin. Biotechnol.* **10**, 579–582 (1999).
5. Dixon, A.K., Richardson, P.J., Pinnock, R.D. & Lee, K. Gene-expression analysis at the single-cell level. *Trends Pharmacol. Sci.* **21**, 65–70 (2000).
6. Brady, G., Barbara, M. & Iscove, N.N. Representative *in vitro* cDNA amplification from individual hemopoietic cells and colonies. *Methods Molec. Cell. Biol.* **2**, 17–25 (1990).
7. Brady, G. & Iscove, N.N. *Methods Enzymol.* **225**, 611–623 (1993).
8. Brady, G. Expression profiling of single mammalian cells—small is beautiful. *Yeast* **17**, 211–217 (2000).
9. Brady, G. *et al.* Analysis of gene expression in a complex differentiation hierarchy by global amplification of cDNA from single cells. *Curr. Biol.* **5**, 909–922 (1995).
10. Billia, F., Barbara, M., McEwen, J., Trevisan, M. & Iscove, N.N. Resolution of pluripotential intermediates in murine hematopoietic differentiation by global cDNA amplification from single cells: confirmation of assignments by expression profiling of cytokine receptor transcripts. *Blood* **97**, 2257–2268 (2001).
11. Brail, L.H. *et al.* Gene expression in individual cells: analysis using global single cell reverse transcription polymerase chain reaction (GSC RT-PCR). *Mutat. Res.* **406**, 45–54 (1999).
12. Cano-Gauci, D.F. *et al. In vitro* cDNA amplification from individual intestinal crypts: a novel approach to the study of differential gene expression along the crypt-villus axis. *Exp. Cell Res.* **208**, 344–349 (1993).
13. Trumper, L.H. *et al.* Single-cell analysis of Reed-Sternberg cells: molecular heterogeneity of gene expression and p53 mutations. *Blood* **81**, 3097–3115 (1993).
14. Robertson, S.M., Kennedy, M., Shannon, J.M. & Keller, G. A transitional stage in the commitment of mesoderm to hematopoiesis requiring the transcription factor SCL/tal-1. *Development* **127**, 2447–2459 (2000).
15. Al-Taher, A., Bashein, A., Nolan, T., Hollingsworth, M. & Brady, G. Global cDNA amplification combined with real-time RT-PCR: accurate quantification of multiple human potassium channel genes at the single cell level. *Yeast* **17**, 201–210 (2000).
16. Liu, F., Malaval, L., Gupta, A.K. & Aubin, J.E. Simultaneous detection of multiple bone-related mRNAs and protein expression during osteoblast differentiation: polymerase chain reaction and immunocytochemical studies at the single cell level. *Dev. Biol.* **166**, 220–234 (1994).
17. Aubin, J.E., Liu, F., Malaval, L. & Gupta, A. Quantitative polymerase chain reaction (PCR): potential applications of poly(A) PCR on samples of limited cell number. *Calcif. Tissue Int.* **56**, S54–S56 (1995).
18. Candeliere, G.A., Rao, Y., Floh, A., Sandler, S.D. & Aubin, J.E. cDNA fingerprinting of osteoprogenitor cells to isolate differentiation stage-specific genes. *Nucleic Acids Res.* **27**, 1079–1083 (1999).
19. Dulac, C. & Axel, R. A novel family of genes encoding putative pheromone receptors in mammals. *Cell* **83**, 195–206 (1995).