Anal Bioanal Chem (2010) 397:1853–1859
DOI 10.1007/s00216-010-3754-0

ORIGINAL PAPER

An improved one-tube RT-PCR protocol for analyzing single-cell gene expression in individual mammalian cells

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Received: 2 March 2010 / Revised: 7 April 2010 / Accepted: 13 April 2010 / Published online: 20 May 2010

Abstract It is well known that gene expression is regulated at the level of individual cells, and more evidence is now emerging that heterogeneity of cell regulation is orders of magnitude greater than previously thought. In order to detect meaningful variations in transcription levels, it is necessary to measure gene expression at single cell levels rather than in bulk cells, where individual differences or heterogeneity could be lost. In this work, we report an improved reverse-transcriptase polymerase chain reaction (RT-PCR) protocol which allows the direct measurement of gene expression in one tube (5–25 μl of total PCR mixture) at the single mammalian cell level. The protocol employs a new cell lysis buffer, and involves no RNA isolation or nested PCR steps, significantly reducing the possibility of contamination and errors. We successfully applied this protocol in qRT-PCR and linear-after-the-exponential (LATE)-PCR to analyze selected genes of various expression levels from three cell lines. Although further characterization of RNA stability is important, the preliminary results showed that gene expression heterogeneity could be common among members of genetically identical cell populations. The protocol illustrated can be utilized for a wide array of applications without much modification, such as cancer cell analysis and preimplantation genetic diagnostics. In addition, the protocol is based on intercalator-based (SYBR Green PCR) chemistry, which is less expensive and suitable for high-throughput platforms.

Keywords Single-cell · Gene expression · One-tube · RT-PCR

Electronic supplementary material The online version of this article (doi:10.1007/s00216-010-3754-0) contains supplementary material, which is available to authorized users.

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Introduction

It has long been accepted that cells in mammalian tissue and genetically identical cell populations are homogeneous at the cell level. However, recent studies have suggested that even cells in a seemingly homogeneous culture or tissue exhibit different characteristics in many respects [1–3]. Some studies have suggested that the heterogeneity of gene expression among individual cells could arise from stochasticity, or noise, in the gene expression of each individual. The amplitude of such noise in gene expression is controlled by many factors, including transcription rate, regulatory dynamics, and genetic factors of the cells [4–9]. As a result of these factors, individual cells in genetically homogeneous populations contain different copy numbers of messenger RNA (mRNA) molecules, which eventually leads to different numbers of functioning protein molecules [9]. In order to better understand the molecular mechanisms that specify the identity and gene-expression palette of any multicellular organism, it is apparent that one needs to address the issues related to gene expression variations from cell to cell. Several methods, such as in situ hybridization and immunohistochemistry, are available to investigate the identity and diverse gene-expression patterns of single cells. However, these methods are mostly semiquantitative and have other limitations, such as difficulty in confirming a probe's specificity, and inability to analyze multiple target genes expressed by the same cell [10].

Conventional polymerase chain reaction (PCR) and reverse-transcript (RT)-PCR are ubiquitous technologies for the direct identification of alleles of specific genes or the mRNA transcribed from those genes. Quantitative RT-PCR (qRT-PCR) offers even better sensitivity and is the gold standard for mRNA quantification [11, 12]. Coupled with various cell sorting and collecting methods, several groups have published protocols for single-cell gene expression analysis by RT-PCR. Most of these studies used relatively large cells, such as embryo [13], retinal ganglion [14], muscle fiber [15] or neuron cells, which are 3–10 times larger than typical mammalian cells [10, 16–18]; or targeted highly expressed alien genes, such as viral genes, in single mammalian cells [18]. In addition, most of these protocols involve either a laborious mRNA purification step or two-stage PCR [10, 12–24]. For RNA isolated from single cells, especially cells of small size, recovery rates have been low, and yield varies significantly from sample to sample, creating problems for accurate and reproducible gene expression measurements [25]. Multiple tube operations increased the chance of contamination when RNA isolation is done separately. For two-stage RT-PCR, although the detection sensitivity is generally improved, false positives become common due to increased PCR cycles. Even for nested PCR, which uses two successive runs of PCR, each with different primers, with the second set intended to amplify a secondary target within the first run product, false positives are still common [26]. This view is confirmed by a recent study demonstrating that the major errors in qPCR came from the pipetting and PCR processes [25]. To address these issues, a single-tube, single-stage RT-PCR protocol needs to be developed. In one recent work, Hartshorn et al. [13] reported a single-tube method for RNA preparation and quantification of Oct4 and Xist templates from large embryo cells using linear-after-the-exponential (LATE)-PCR, an advanced form of asymmetric PCR for real-time measurement. This study employed complementary methods to confirm LATE-PCR's specificity and quantitative accuracy.

In this work, we present an improved one-tube, single-cell RT-PCR analysis of gene expression in individual cells of three mammalian cell lines without RNA purification and nested PCR. The lysis buffer in the protocol, RNaST, exhibits minimal inhibitory effects on the RT-PCR, so that we can decrease the RT-PCR volume to 5 μl. The single-cell lysate, obtained by quick freezing on dry ice and subsequent thawing in RNaST, was reverse transcribed and quantitatively amplified in one tube. Gene expression analysis by this protocol showed that the heterogeneity among cells can be lost when even as few as five or ten cells are used in one assay. We also successfully applied this protocol for the LATE-RT-PCR analysis of single-cell gene expression.

Materials and methods

Cell lines

Human lung epithelial carcinoma A549 cells were purchased from the American Type Culture Collection (ATCC) and maintained in Ham's F12-K medium (Invitrogen, Carlsbad, CA, USA), with 10% fetal calf serum and 5% penicillin/streptomycin (Sigma, St. Louis, MO, USA). Barrett's epithelial CP-C cells [27, 28] were kindly provided by Dr. Brain Reid, Fred Hutchinson Cancer Research Center, Seattle, Washington, and were cultured in keratinocyte-serum free medium supplemented with bovine pituitary extract (BPE) and human recombinant epidermal growth factor (rEGF) (Invitrogen, Carlsbad, CA, USA). Human cervical cancer HeLa cells received from ATCC were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and antibiotics.

Single-tube sample collection and cell lysis

Mammalian cells were diluted to 10^2–10^3 cells per ml in phosphate buffer (PBS) (pH 6.8). Cell picking was done using a robotic single-cell manipulation system developed in our research center [29], which can aspirate a single cell in a total volume of 50 nl. The single cell aspirated was delivered onto the lid of a regular 100 μl PCR tube (Applied Biosystems, Foster City, CA, USA), containing 2 μl of denaturing reagents (RNaST lysis buffer). After closing the lid, the tube was centrifuged briefly in a "Quick-Spin" Minifuge (ISC BioExpress, Kaysville, UT, USA) to let the cell and RNaST lysis buffer [RNaST lysis buffer: RNase inhibitor: 2 μ/μl (Ambion, Austin, TX, USA); NaCl: 0.135 M; Tris-HCl (pH 8.0): 9 mM; dithiothreitol: 4.5 mM] settle at the bottom of the tube. The tube was frozen on dry ice immediately, and then stored at −80 °C for further gene expression analysis. Alternatively, the cell suspension was subjected to serial dilution with sterile 1xPBS to achieve a theoretical given cell number in a final total volume of 2.5 μl. The solution with cells (2.5 μl) was then transferred to a 0.2 ml (or 0.1 ml) PCR tube containing 2.5 μl of RNaST lysis buffer. The cells were then quickly frozen on dry ice and stored at −80 °C.

Genes selected for single-cell expression analyses

Three genes were selected for single-cell analysis: glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank Access No. NM_002046), cell cycle regulator gene p16 (CDKN2A, GenBank Access No. NM_000077), and tumor suppressor gene p53 (GenBank Access No. NM_000546). These genes are expressed at different levels in mammalian

cells. Primers for these genes were designed using VisualOMP (DNA Software, Ann Arbor, MI, USA) and synthesized by BioSearch Technologies (Novato, CA, USA). The amplified PCR fragment was designed to span two exons of the genes of interest in order to eliminate genomic DNA contamination. The primers and probes used in this study are listed in Table 1. The RT-PCR for series diluted cells was run according to the following thermal profile: 1 cycle at 45 °C for 45 min; 1 cycle at 95 °C for 2 min; 60 cycles at 95 °C for 20 s, 65 °C for 20 s, and 68 °C for 30 s; a final extension at 68 °C for 7 min and a soak at 4 °C were used. The RT-PCR cycling condition for picked single cells was run in a Corbett Research RG-6000 using the SuperScript III Platinium SYBR Green One-Step qRT-PCR kit from Invitrogen: 1 cycle at 45 °C for 30 min; 1 cycle at 95 °C for 2 min; 50 cycles at 95 °C for 15 s, 60 °C for 30 s; 1 cycle at 40 °C for 1 min following melting curve analysis according to the instrument documentation. After the reverse transcription reaction, LATE-PCR was performed in a Corbett Research RG-6000 by adding the appropriate reagents in the same vessel. Fragments of the genes of interest were amplified using the total cDNA from 5 μl of reverse transcription in single-cell lysate as the template. Each reaction was run in a final volume of 25 μl and contained the following reagents: 2.5 μl of 10x reaction buffer, 2.5 μl of 2 mM dNTPs, 1.5 μl of 50 mM $MgCl_2$, 5 μl of RT reaction mixture, 1 μl of 25 μM excess primer, 1 μl of 1.25 nM limit primer, 1 μl of 25 μM probe, 1 μl of 7.5 μM PrimeSafe 001, 1 μl of 0.625 μM PrimeSafe 060 [13], 0.25 μl of Platinum Tag from Invitrogen, and 7.25 μl of DNase/RNase free water. The PCR profile was 1 cycle at 95 °C for 3 min; 15 cycles at 95 °C for 10 s, 72 °C for 20 s; and 50 cycles at 95 °C for 10 s, 72 °C for 10 s, 68 °C for 10 s, 72 °C for 20 s and 60 °C for 20 s, with fluorescence acquisition at 60 °C following the melting program from 50 °C to 80 °C. This additional melting program will evaluate whether the PCR program generates a single PCR product band. After RT-PCR, 10 μl of the PCR mixture were analyzed by electrophoresis in a 2% agarose gel containing SYBR Green prior to being visualized under UV light with the Typhoon Troi image system (GE Healthcare).

Table 1 Oligos and probe used in the study

Name of oligo	Sequences	GenBank No
hGAPDH-F	5′-AAGGTCGGAGT CAACGGATTTGGT-3′	NM_002046
hGAPDH-R	5′-AGTGATGGCATGG ACTGTGGTCAT-3′	
hp53-F	5′-CTTCTGTCCCTTCC CAGAAAACCTACC-3′	NM_000546
hp53-R	5′-AGACTTGGCTGTC CCAGAATGCAAG-3′	
hp53-lim	5′-ACTGTCCCAGAATGC AAGAAGCCCAGACG-3′	NM_000546
hp53-exc	5′-TGTCATCTTCTGTCC CTTCCCAGAAA-3′	
hp53-Pr	5′ BHQ-1 TACCAGAATG CAAGAAGCCCATA-3′ Cal Orange 560	

Results and discussion

RNaST as a good lysis buffer

Detergents such as SDS in cell lysis buffers strongly inhibit the PCR, so that the initial lysate needs to be diluted to ensure that the PCR (or RT-PCR) works properly. The increased volume of PCR negatively affects the sensitivity for gene expression in single cells. To address this issue, we started by evaluating several commercial lysis buffers, including Celcytic M cell lysis buffer from Sigma, Glo lysis buffer from Promega, and the RNaST buffer we developed. The effect of inhibition was investigated by adding various amounts of the lysis buffer under test to the PCR in a total volume of 25 μl. The results showed that Celcytic cell lysis buffer inhibited the PCR at 10% (v/v) of total volume, and Glo cell lysis buffer inhibited the PCR at 4% (v/v) of the total volume (data not shown). RNaST lysis buffer showed no inhibition when 12% (v/v) of RNaST was placed in a PCR tube of 25 μl total volume. Significant inhibition was observed only when RNaST constituted more than 25% (v/v) of the total volume (Fig. 1). Since the results suggested that RNaST could be a good lysis buffer in our efforts to develop one-tube RT-PCR, we selected it for further study.

One-tube single-cell RT-PCR analysis

Two types of quantitative PCR approaches are available. One is probe-based (TaqMan PCR) and the other is intercalator-based (SYBR Green PCR). In general, the TaqMan method is considered more accurate and reliable than the SYBR green method, but is also much more expensive. Since our goal is to develop methods that can potentially be used for high-throughput analysis, we chose the SYBR green method as an inexpensive platform to start with. RT-PCR analyses for p53, p16 and GAPDH in A549 cells were performed. These are genes with different lengths and different expression levels. First, we used series dilution to generate cell solutions containing 1,000 cells to 1 cell in each PCR tubes. The frozen cells in the PCR tube were lysed in the RNaST lysis buffer when the tube was quickly frozen on dry ice and subsequently thawed at room temperature. The entire cell lysate (5 μl total) was used as the PCR template in a final volume of 25 μl mixture. RT-PCR was carried out in an ABI GeneAmp PCR System 9700 (Applied Biosystems, Foster

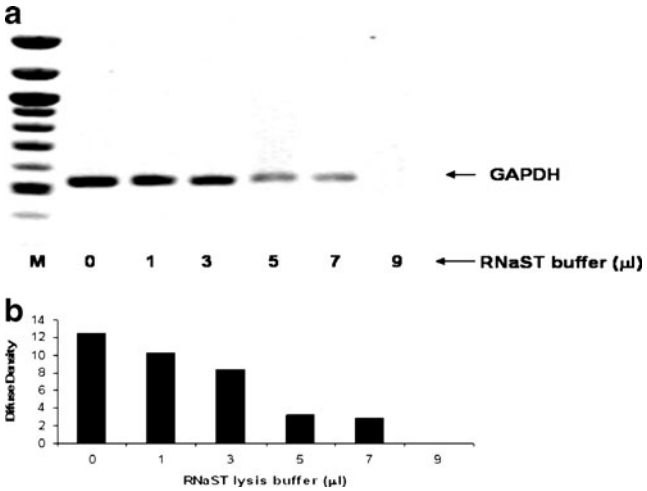


Fig. 1 Inhibitory effect on RT-PCR of RNaST lysis buffer. **A** Gel electrophoresis analysis of RT-PCR containing varying amounts of RNaST buffer in a 25 μl PCR mixture on GAPDH gene transcripts by purified RNA; **B** Densitometry assay for panel **A**. 100 bp ladder was used

City, CA, USA) using the One-Step RT-PCR kit from Promega (Madison, WI, USA). The series dilution results showed that clear bands of the correct size were observed at the single-cell level for p53, and at the three- to five-cell level for GAPDH and p16 genes (data not shown).

We further conducted experiments to test whether the same protocol can be used in qRT-PCR for direct single-cell detection. In this experiment, single cells were picked by a robotic micromanipulator [29] and aspirated onto 0.1 ml PCR tube lids containing 2 μl RNaST lysis buffer, followed by spinning down in the minifuge. After quick freezing on dry ice and subsequent thawing at room temperature, the entire 2 μl of cell lysate was used as template for amplification in a 25 μl mixture. We evaluated the detection of p53 gene expression in Barrett's esophagus CP-C cells and GAPDH transcript in A549 cells at the single-cell level. The results showed that expression of the p53 gene was detected in three out of four tubes containing a single cell with a standard deviation of Ct value of around 2.75 (Fig. 2). For the one that was undetected due to its low Ct value, the corresponding band of the correct size was seen on agarose gel (data not shown). GAPDH gene expression in single-cell samples was detected in five cells with a standard deviation of Ct value of around 0.91, although two negative controls in GAPDH transcript analysis showed some residual signal (Fig. 3). Single-cell level gene expression detection was also achieved for p53 gene transcript in HeLa cells (Fig. 4). The results demonstrated that by performing quick freezing and subsequent thawing in RNaST buffer we can achieve one-tube qRT-PCR analysis of single mammalian cells without RNA isolation or two-stage nested PCR.

LATE-PCR is an approach that combines the efficiency of exponential amplification in the early phases of the reaction with the advantages of linear amplification thereafter [30, 31]. LATE-PCR increases PCR sensitivity, and provides a suitable method to detect genes with a single allele [32, 33]. LATE-PCR technology was recently modified to create LATE-RT-PCR for increased detection sensitivity to genes in single embryo cells [13]. We also tested our protocol for p53 gene expression in single A549 cells by LATE-RT-PCR. Single cells were picked by robotic micromanipulator and loaded into 2 μl RNaST lysis buffer as described above. After quick freezing on dry ice and subsequent thawing at room temperature, the entire 2 μl cell lysate was used as the template for reverse transcription. All RT reagents were from Qiagen (Valencia, CA, USA) and were used at the suggested concentrations with oligo-dT as primer, but the total volumes were reduced by 75% since the assay was performed in just 5 μl. The result showed that p53 gene expression was detected in seven of eight single cells with a standard deviation of Ct value of around 2.55 (Fig. 5), proving that our one-tube protocol can be integrated with LATE-RT-PCR for single-cell gene expression analysis.

As one of our long-term goals is to develop single-cell analytical methods that can be applied in a high-throughput manner, we sought to determine the minimal total volume required for reproducible qPCR measurements. Reducing the total volume used in the qRT-PCR decreases the cost and could potentially increase RNA template concentration in the reaction mixture, increasing the sensitivity of single-cell analysis. In the study described above, we reduced the total volume of PCR to 20 to 25 μl from the typical 100 μl. To test whether the protocol can be used with smaller volumes, we first used different amounts of the positive control kanamycin resistance gene mRNA with carrier as template and specific

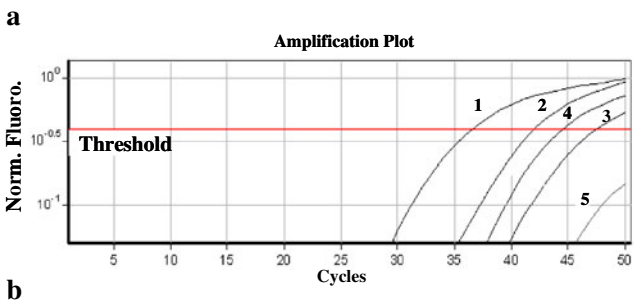
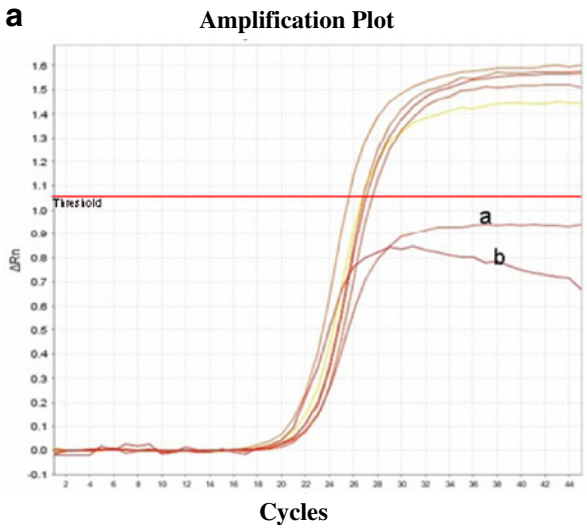


Fig. 2 p53 gene transcript in single-cell qRT-PCR in CP-C cells. **A** Real-time RT-PCR running curve in four single cells plus positive and negative controls; **B** Corresponding Ct values for samples in panel **A**. The default threshold was applied



b

Sample	Ct
Single cell 1	25.34
Single cell 2	26.87
Single cell 3	26.96
Single cell 4	27.25
Single cell 5	27.78
Negative control 1	N/A
Negative control 2	N/A

Fig. 3 GAPDH transcript by qRT-PCR in single individual cells of A549. **A** Real-time PCR running plots for single cells. *a* and *b* indicate negative controls with no cells. The others represent data for single-cell qRT-PCR without RNA isolation/purification; **B** The five single cells and two negative controls along with their corresponding Ct values in panel **A**. The default threshold was applied

target sequence primers from the Access RT-PCR System (Promega, Madison, WI, USA) to perform qRT-PCR in various total volumes. The results indicated that there were no significant differences in detection sensitivity for total volumes of 20 μl, 10 μl, 5 μl, or 2 μl (see the "Electronic supplementary material," Fig. S1). We then investigated the minimal volume needed for one-tube qRT-PCR analysis with single HeLa cells, targeting p53 gene transcript. The results showed that reproducible measurements were obtained even when the total PCR volume was reduced to 5 μl ("Electronic supplementary material," Fig. S2). No satisfactory measurements were obtained when the total volume was reduced to 2 μl (data not shown).

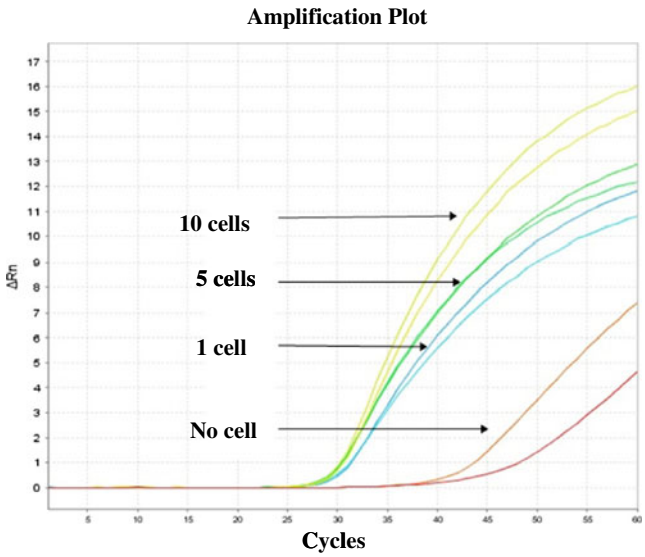


Fig. 4 qRT-PCR analysis of p53 from different numbers of HeLa cells. Each curve represents a single detection of a p53 transcript, where the number of cells used is indicated. Two negative controls are shown in *red*

Single-cell qRT-PCR analysis reveals gene expression heterogeneity

To demonstrate that the protocol can be used to reveal gene expression heterogeneity among cells, we isolated single cells from an isogenetic HeLa cell population and used them for qRT-PCR gene expression analysis. We performed qRT-PCR analysis of p53 gene transcript with ten, five or one cell in each PCR tube (Fig. 6). All cells were individually picked using the robotic micromanipulator. The results showed that the measurements from the single-cell tubes had the widest variation in expression among the tubes, with a standard deviation of Ct value of 5.75, while the measurements for ten cells per tube or five cells per tube gave much less variation among tubes, with standard deviation of Ct values of 1.79 and 1.65, respectively—only 31% and 29% of the variation found among PCR tubes with a single cell (Fig. 6d). Measurements of standard deviations and Ct span values (maximal Ct to minimal Ct) showed similar trends (Fig. 6d). The results were consistent with early suggestions that it is necessary to develop a quantitative protocol to measure gene expression in single eukaryotic cells rather than in the bulk, where gene expression heterogeneity will be masked by averaging effects.

Conclusion

The technologies developed for single-cell manipulation have led to new assays for the molecular biology, cell biology and physiology of single cells [29, 35–37]. The analysis of gene expression profiles in individual cells is required to further our understanding of human disease at the cellular level and to gain fundamental knowledge of mechanisms in cellular transduction, differentiation, development and malignant transformation [34]. In this paper, we report an improved one-tube qRT-PCR protocol for

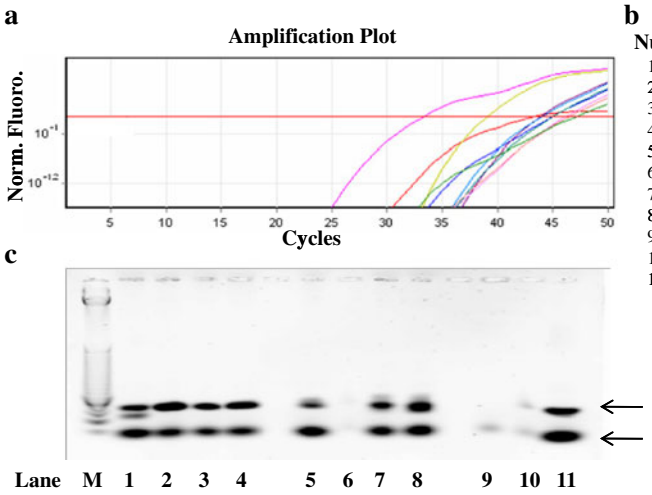
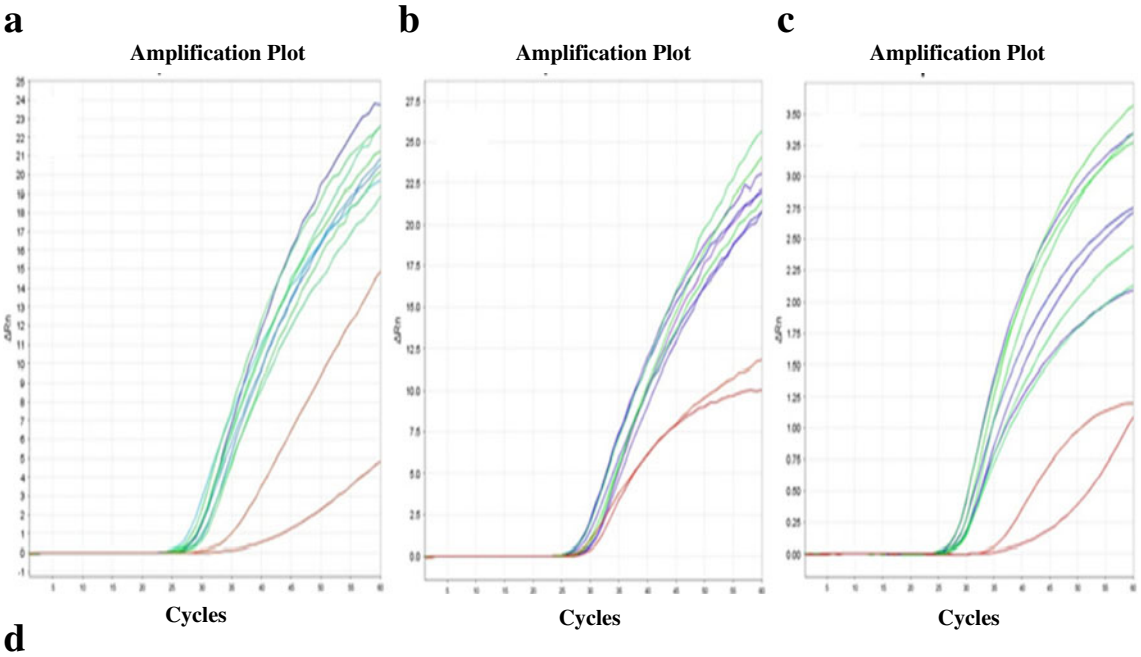


Fig. 5 p53 gene transcript by single-cell LATE-RT-PCR in A549 cells. **A** Real-time PCR running plot; purified RNA is used as positive control. **B** Ct values of eight single cells, two negative controls and one positive control; **C** gel electrophoretic analysis of the LATE-RT-PCR mixtures in panels **A** and **B**. Typical two-band LATE-PCR products are indicated by *arrows*. The default threshold was applied. 100 bp ladder was used



d

No of cells	No of samples	Average (Ct)	STDEV *	SE **	Span ***
10	10	44.52	1.7907	0.5969	5.53
5	8	45.75	1.6508	0.6240	4.26
1	9	44.29	5.7493	2.0327	14.36

"*" Standard deviation;
"**" Standard Error;
"***" Maximal Ct value – minimal Ct value in the detected samples

Fig. 6 Gene expression heterogeneity in a genetically identical cell population. qRT-PCR analysis of p53 transcript in HeLa cells with two negative controls in *red*. **A** qRT-PCR analysis of p53 transcript with ten cells in each PCR. Ten PCRs were analyzed. **B** qRT-PCR analysis of p53 transcript with five cells in each reaction. Eight PCRs were analyzed. **A** qRT-PCR analysis of p53 transcript with a single cell in each reaction tube. Nine reactions were analyzed. **D** Statistical analysis of Ct values from panels **A**, **B**, and **C**. The final total volume was 25 μl for all PCR analyses

analyzing single-cell gene expression in individual, small mammalian cells. Quick freezing and thawing in an RNaST lysis buffer proved to be an effective method for cell lysis, and the lysate exhibited minimal inhibitory effects on the RT-PCR. Advantages of this method include:

- *One-tube operation*. RT-PCR and qRT-PCR are highly sensitive techniques, and any level of contamination will result in false results, especially when the template concentration is low, as it is for single cells. Our protocol allows qRT-PCR to be performed in one tube from single-cell loading, cell lysis, reverse transcription to PCR amplification, reducing the risk of contamination, template loss, and major errors from the pipetting and PCR processes [25].
- *High sensitivity and lack of need for a two-stage PCR*. Because inhibition by RNaST lysis buffer on RT-PCR is pretty minimal, our protocol ensures that the RT-PCR is highly efficient by reducing the final RT-PCR volume to 25 μl to enhance the template concentration relative to the PCR mixture, while most other current protocols use final PCR volumes of 50 μl or even 100 μl [13, 32]. They also require another stage of nested PCR [20, 23], which results in a significantly increased chance of false results resulting from both human error and the PCR process.
- *High reliability and easy of use.*
- *Inexpensive SYBR Green chemistry*, which is suitable for high-throughput platforms.

Because of the snapshot nature of the qRT-PCR method, the cell-to-cell difference we observed here could also be due to mRNA molecule instability. Although further characterization of the stability of mRNA is important, measurements of p53 gene transcript using this protocol provided preliminary evidence that gene expression heterogeneity among individual cells could be significant, and that the application of the single-cell gene expression assay will be helpful for studying cell heterogeneity.

Acknowledgements We would like to thank Drs. L. Wangh and A. Sanchez from Brandies University for their help with LATE-PCR technology. We would also like to thank Dr. B. Reid for kindly supplying the CP-C cells. Dr. Y. Anis is acknowledged for his help with single-cell loading. This work was supported by a CEGS Microscale Life Science Center (MLSC) project (Grant #: p50-HG002360) from NIH, USA.

References

1. Eddinger TG, Meer DP (1997) Comp Biochem Physiol 117B:29–38
2. Lidstrom ME, Meldrum DR (2003) Nat Rev Microbiol 1:158–164
3. Jensen KB, Watt FM (2006) Proc Natl Acad Sci USA 103:11958–11963
4. Ross IL, Browne CM, Hume DA (1994) Immunol Cell Biol 72:177–185
5. Elowitz MB, Levine AJ, Siggia ED, Swain PS (2002) Science 297:1183–1186
6. Blake WJ, Kaern M, Cantor CR, Collins JJ (2003) Nature 422:633–637
7. Cai L, Friedman N, Xie XS (2006) Nature 440:358–362
8. Maheshri N, O'Shea EK (2007) Ann Rev Biophys Biomol Struct 36:413–434
9. Raj A, Peskin CS, Tranchina D, Vargas DY, Tyagi S (2006) PLoS Biol 4:e309
10. Esumi S, Kaneko R, Kawamura Y, Yagi T (2006) Nat Protoc 1:2143–2151
11. Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonak J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N (2006) Mol Aspects Med 27:95–125
12. Nolan T, Hands RE, Bustin SA (2006) Nat Protoc 1:1559–1582
13. Hartshorn C, Eckert J, Hartung O, Wangh L (2007) BMC Biotechnol 7:87
14. Lindqvist N, Vidal-Sanz M, Hallbook F (2002) Brain Res Protoc 10:75–83
15. Wacker MJ, Tehel MM, Gallagher PM (2008) J Appl Physiol 105:308–315
16. Eberwine J, Yeh H, Miyashiro K, Cao Y, Nair S, Finnell R, Zettel M, Coleman P (1992) Proc Natl Acad Sci USA 89:3010–3014
17. Christensen KV, Lambert DWM, JDC EJ (2000) NeuroReport 11:3577–3582
18. Esumi S, Wu SX, Yanagawa Y, Obata K, Sugimoto Y, Tamamaki N (2008) Neurosci Res 60:439–451
19. Faumont N, Saati TA, Brousset P, Offer C, Delson G, Meggetto F (2001) J Gen Virol 82:1169–1174
20. Miragi T, Murakami K, Sawada T, Taguchi H, Miyoshi I (1998) Leukemia 12:1645–1650
21. Liss B (2002) Nucleic Acids Res 30:e89
22. Yan L, Kaczorowski G, Kohler M (2002) Anal Biochem 304:267–270
23. Vrettou C, Traeger-Synodinos J, Tzetis M, Palmer G, Sofocleous C, Kanavakis E (2004) Hum Mutat 23:513–521
24. Peixoto A, Monteiro M, Rocha B, Veiga-Fernandes H (2004) Genome Res 14:1938–1947
25. Taniguchi K, Kajiyama T, Kambara H (2009) Nat Meth 6:503–506
26. Szöllős E, Hellgren O, Hasselquis D (2008) J Parasitol 94:562–564
27. Polanca-Wessels MC, Barrett MT, Galipeau PC, Rohrer KL, Reid BJ, Rabinovitch PS (1998) Gastroenterology 114:295–304
28. Palanca-Wessels MCA, Klingelhutz A, Reid BJ, Norwood TH, Opheim KF (2003) Carcinogenesis 24:1183–1190
29. Anis YH, Holl MR, Meldrum DR (2008) Automated vision-based selection and placement of single cells in microwell array formats. In: Proc 4th IEEE Conf Automation Science and Engineering, Washington, DC, USA, 23–26 Aug 2008, p 315
30. Sanchez JA, Pierce KE, Rice JE, Wangh LJ (2004) Proc Natl Acad Sci USA 101:1933–1938
31. Pierce KE, Sanchez JA, Rice JE, Wangh LJ (2005) Proc Natl Acad Sci USA 102:8609–8614
32. Hartshorn C, Anshelevich A, Wangh LJ (2005) BMC Biotechnol 5:2
33. Salk JJ, Sanchez JA, Peirce KE, Rice JE, Soares KC, Wangh LJ (2006) Anal Biochem 353:124–132
34. Phillips JK, Lipski J (2000) Auton Neurosci 86:1–12
35. McGuire S, Fisher C, Holl MR, Meldrum DR (2008) Rev Sci Instrum 79:086111
36. Nandakumar V, Holl MR, Meldrum DR (2008) A flexible framework for automation of single cell and cell-to-cell interaction analyses. In: Proc 4th IEEE Conf Automation Science and Engineering, Washington, DC, USA, 23–26 Aug 2008, p 424
37. Chao SH, Meldrum DR (2009) Lab Chip 9:867–869