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# Measurement of Single-Cell Gene Expression Using Capillary Electrophoresis

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**Capillary electrophoresis with laser-induced fluorescence detection was used to monitor gene expression in individual mammalian cells using the reverse transcriptase-polymerase chain reaction. Specifically, $\beta$-actin expression in single LNCaP (prostate cancer) cells was measured. A sieving matrix containing hydroxypropyl methyl cellulose was used to effect size-based separation. Ethidium bromide fluorescence of the product DNA was used as the detection scheme and yielded excellent sensitivity. The $\beta$-actin product, resulting from an individual cell lysed by a freeze−thaw method, gave an average signal-to-noise ratio (S/N) of 77 ± 27 ($n$ = 2). Chemical lysis of a single cell, using a dilute solution of SDS, gave a S/N of 26 ± 2 ($n$ = 2), roughly 3-fold lower than for freeze−thaw lysis. An initial detection limit (not considering fully optimized conditions) was calculated from an amplified cDNA standard to correspond to a concentration of ∼133 starting molecules/nL (of $\beta$-actin mRNA).**

The measurement of gene expression is commonly performed using the reverse transcriptase-polymerase chain reaction (RT-PCR) method.[1−3] When a gene is expressed in vivo, a DNA sequence is transcribed to form a strand of messenger RNA (mRNA), and the mRNA is subsequently translated into a polypeptide chain. In RT-PCR, the mRNA produced by the specific gene sequence is reverse transcribed to form complementary DNA (cDNA), and that cDNA is amplified using PCR. Practical aspects of this methodology typically include isolation of RNA from the desired population of cells and detection of the resultant DNA product with an electrophoresis method.[3]

The first demonstration of single-cell RT-PCR (SC-RT-PCR) was reported in 1989 by Rappolee et al.[4] Since then, RT-PCR has become an increasingly popular method for monitoring gene expression of single cells, especially in neurochemistry. SC-RT-PCR of neurons is often coupled with the electrophysiology method of patch-clamping, because the intracellular contents can be easily aspirated into the patch-clamp pipet after recording, for subsequent expulsion into a reaction mixture (representative references are cited).[5−23] Intracellular contents from cells have also been aspirated using a patch-clamp setup without actual patch-clamp recording.[12,24−28] Other techniques for single-cell isolation and sampling include using a micropipet to isolate an intact cell for subsequent lysis[29,30] and using flow-sorting[31,32] or dilution[33] to

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place cells into wells for centrifugation and/or lysis. SC-RT-PCR has also been applied to non-neuronal cells. Examples of such applications include the analysis of kidney cells,[28] hemopoietic cells,[34] B-cell hybridomas,[35] T-cells,[36] and macrophages.[4] In addition, diagnostic applications[37] and early development of oocytes and embryos[38−40] have been examined.

Detection and identification of SC-RT-PCR products is typically accomplished using slab gel electrophoresis. Although CE is finding a place for bulk (i.e., non-single-cell) RT-PCR analysis,[41,42] it is virtually nonexistent for single-cell applications, despite its inherent sensitivity and low volume requirements. To date, the only study in which CE has been used to detect SC-RT-PCR products involved very large cells (i.e., oocytes) or embryos as samples.[39]

In this paper, we report the first use of capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) to detect a SC-RT-PCR product, using small mammalian cells (i.e., ~10 $\mu$m), to monitor expression of $\beta$-actin. We describe a simple and rapid cell-lysis method using either dilute surfactant, as commonly used for single-cell lysis,[43−46] or a freeze−thaw approach to gain access to the cellular interior.

## EXPERIMENTAL SECTION

**Chemicals and Reagents.** Hydroxypropyl methyl cellulose (HPMC-5) with a molecular mass of 10 000 was purchased from Aldrich (Milwaukee, WI). Poly(vinylpyrrolidone) (PVP) was obtained from Polysciences (Warrington, PA). Mannitol, sodium dodecyl sulfate (SDS), and tris(hydroxymethyl)aminomethane (Tris) were from Sigma (St. Louis, MO). Trypsin-EDTA (0.25%) and ethylenediaminetetraacetic acid disodium salt (EDTA) were from Life Technologies (Gaithersburg, MD). ΦX174/*Hae*III DNA, containing 11 double-stranded fragments (72, 118, 194, 234, 271, 281, 310, 603, 872, 1078, and 1353 bp), was purchased from Promega (Madison, WI). Ethidium bromide was from Molecular Probes (Eugene, OR). Molecular biology grade water (i.e., RNase/DNase-free), which was used to make all solutions, was purchased from Biowhittaker (Walkersville, MD). Gene-specific primers for human $\beta$-actin (sense, 5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′; antisense, 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′) were obtained from Clontech (Palo Alto, CA). All other reagents used for RT-PCR were from Promega.

**Cell Preparation.** A human prostate carcinoma (LNCaP) cell line, obtained from American Type Culture Collection (ATCC, Manassas, VA), was used for all single-cell RT-PCR experiments. Cells were cultured in RPMI-1640 medium (ATCC) supplemented with 10% fetal bovine serum, 0.18 $\mu$g/mL streptomycin, and 0.18 IU/mL penicillin (all from Life Technologies). Cells were grown in an incubator set at 37 °C with a humid atmosphere of 5% $CO_2$ and prepared for use via a previously described protocol.[45]

**Microvial Construction.** RT-PCR microvials (~30-$\mu$L final volume) were constructed using a procedure based on that of Page et al.[47] In our approach, a lighter was used to seal the ends of sterile 1−200-$\mu$L pipet tips (Fisher Scientific, Fairlawn, NJ). The tips were then cut approximately 1.5−2 cm from the sealed end to form the reaction vial. Following introduction of RT-PCR components, the vials were sealed with a lighter.

**Single-Cell Introduction.** A device was constructed to isolate an intact cell and deliver it or its lysate to 5-$\mu$L aliquots of the RT-PCR mixture. The setup consisted of a 12-cm segment of fused-silica capillary (50-$\mu$m i.d., 360-$\mu$m o.d.; Polymicro) attached to a 5-mL syringe containing sieving buffer (see below), via a short piece of plastic tubing. The polyimide coating was removed from the capillary tip with a lighter for clearer viewing of cell retrieval. The capillary was anchored to a Micromaster III microscope (Fisher Scientific), using a special mounting device, such that the tip was resting on a slide within the field of view. A 20-$\mu$L aliquot of cells was placed over the capillary tip, and an individual cell was suctioned into the capillary. For cells being chemically lysed, a droplet of 0.05% w/v SDS was also placed on the slide. Once the cell was in view, the slide was moved such that the capillary tip rested in the SDS solution. A small plug of SDS was pulled into the capillary, and lysis was visually confirmed. The contents of the capillary were then expelled into the microvial containing the RT-PCR mixture. For cells being lysed using a freeze−thaw cycle, the whole cell was expelled into the reaction mixture. The tubes were sealed and placed in dry ice for 5 min. Following lysis, all tubes were stored on ice until placed into the thermal cycler.

**RT-PCR Master Mixture.** A single-tube RT-PCR master mixture was prepared by combining the following in a sterile, 200-$\mu$L vial: (a) 56 $\mu$L of molecular biology grade water; (b) 20 $\mu$L of AMV/*Tfl* 5× reaction buffer; (c) 2 $\mu$L of PCR nucleotide mix (10 mM each dNTP in water); (d) 5 $\mu$L of each human $\beta$-actin primer (20 $\mu$M each primer); (e) 4 $\mu$L of $MgSO_4$ (25 mM); (f) 2 $\mu$L of *Tfl* DNA polymerase (5 IU/$\mu$L); and (g) 2 $\mu$L of RNasin ribonuclease inhibitor (40 IU/$\mu$L). A 10-$\mu$L aliquot of this mixture was placed in a separate vial to allow control experiments to be performed in the absence of reverse transcriptase. To the remaining mixture, 0.8 $\mu$L of AMV reverse transcriptase (10 IU/$\mu$L) was added. The master mixture was prepared daily and stored on ice.

**RT-PCR Protocol.** A schematic that summarizes the experimental protocol (cell introduction, lysis, RT-PCR, and CE-LIF) is shown in Figure 1. All RT-PCR experiments, except the positive control, were performed in the 30-$\mu$L vials described above. For single-cell RT-PCR, an individual cell was added to a 5-$\mu$L aliquot of the RT-PCR mixture. For experiments performed in the absence of reverse transcriptase, 5 $\mu$L of the RT-PCR mixture without reverse transcriptase was substituted. For positive-control RT-PCR, 0.5 $\mu$L of positive control cDNA (50 amol/$\mu$L; Clontech) was


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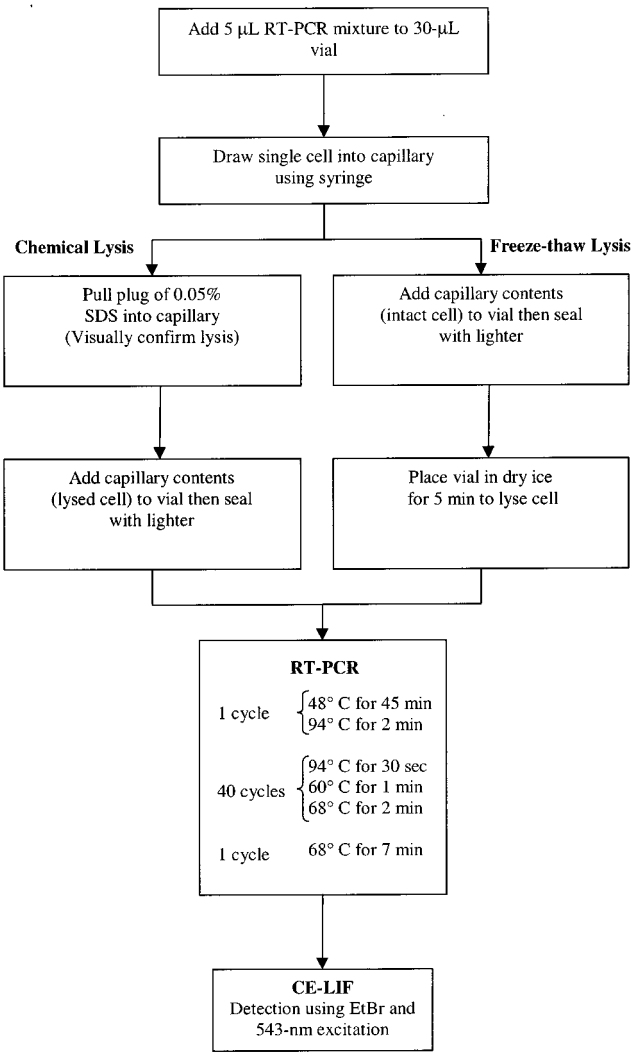


Figure 1. Flowchart showing methods used to perform single-cell RT-PCR and CE-LIF analysis.

combined with 12 $\mu$L of the RT-PCR master mix in a 200-$\mu$L thin-walled PCR tube. Negative control RT-PCR was performed by combining ~200 nL of sieving buffer (see below) with 5 $\mu$L of RT-PCR master mix. RT-PCR was performed in a PCT-200 DNA Engine thermal cycler (MJ Research, Waltham, MA). The reverse transcription step was performed using 1 cycle of 48 °C for 45 min followed by 1 cycle of 94 °C for 2 min. PCR was performed directly following reverse transcription (in the same tube) using the following protocol: 40 cycles of 94 °C for 30 s (denaturation), 60 °C for 1 min (annealing), 68 °C for 2 min (extension); and 1 cycle of 68 °C for 7 min (final extension).

**CE-LIF System.** Analysis of RT-PCR products was performed on a laboratory-built CE-LIF system, which has been described previously.[44] Separation was performed using 40-cm (25-cm effective length) bare fused-silica capillaries (50-$\mu$m i.d., 360-$\mu$m o.d.) from Polymicro Technologies (Phoenix, AZ). The sieving matrix used for separation has been described previously[44] and contained 1% HPMC, 0.5% PVP, and 6% mannitol dissolved in TBE buffer (100 mM Tris−100 mM boric acid−2 mM EDTA, pH 8.3). Ethidium bromide, a DNA intercalator, was added such that the final concentration was 0.5 $\mu$g/mL. A 4-mW green helium−neon laser (JDS Uniphase Inc., Manteca, CA) provided a 543.5-nm excitation source. To drive the electrophoresis, a positive potential (+7.8 kV) was applied to the outlet and the inlet was grounded. All samples and standards were electrokinetically injected for 5 s at 7.8 kV. Data were digitized using a DT 2804 data acquisition board, and electropherograms were generated using ChromPerfect software (Justice Innovations, Mountain View, CA). The original data files were converted to ASCII and replotted using Excel.

## RESULTS AND DISCUSSION

**Data Analysis.** For our initial demonstration of single-cell RT-PCR with CE, a gene-specific primer pair for human $\beta$-actin, which should give an 838-bp product, is used in conjunction with LNCaP cells. Figure 2 shows an electropherogram of ΦX174 DNA/*Hae*III markers and a positive control electropherogram, using the manufacturer-provided 838-bp DNA fragment as a PCR template (inset). The set of peaks centered at ~14 min results from primer dimer formation during PCR. These types of primer aggregates are often seen with PCR[48] and are due to self-annealing of excess primers. The second peak, with a migration time of 19.44 min, is the DNA product peak. When qualitatively compared with the electropherogram of the ΦX174 DNA/*Hae*III markers, the product peak falls in the expected position between the 603- and 872-bp fragments.

Representative single-cell RT-PCR electropherograms are shown in Figure 3. Single-cell RT-PCR was performed using both freeze−thaw lysis (Figure 3A) and SDS lysis (Figure 3B). As with the positive control, the electropherograms from single-cell RT-PCR show a set of primer dimer peaks and an isolated peak at ~19.40 min. A comparison with the positive control electropherogram (Figure 2 inset) indicates that this isolated peak from the single-cell runs corresponds to the expected 838-bp product.

Other control runs were also performed and are shown in Figure 4. A negative control run, in which sieving buffer was substituted for the cell, yielded no product other than primer dimers (Figure 4A). A control run in the presence of a freeze−thaw lysed cell, but in the absence of reverse transcriptase, yielded no product either (Figure 4B). This confirms that the 838-bp product is the result of cDNA amplification and not the result of genomic DNA amplification or simply an anomalous peak.

**Comparison of Lysis Methods.** Various methods of cell lysis were examined for their effectiveness. Lysis conditions must be carefully chosen so that complete lysis is achieved quickly and reproducibly without destroying the enzymes used in RT-PCR (*AMV reverse transcriptase and Tfl DNA polymerase*). Freeze−thaw lysis, in which the cell is frozen at −70 °C and then allowed to thaw, has been used in RT-PCR studies with whole cells.[23,29,49] Because no foreign chemicals are introduced, potential interferences with RT-PCR components are avoided. The electropherogram in Figure 3A resulted from SC-RT-PCR using this method of lysis. The average signal-to-noise ratio (S/N) using freeze−thaw lysis was 77 ± 27 ($n$ = 2). Chemical lysis, using a dilute solution of SDS, was performed to provide a mode of comparison to the freeze−thaw method. The electropherogram in Figure 3B resulted from SC-RT-PCR using a 0.05% w/v SDS solution for lysis. This method gave a S/N of 26 ± 2 ($n$ = 2), roughly 3-fold lower

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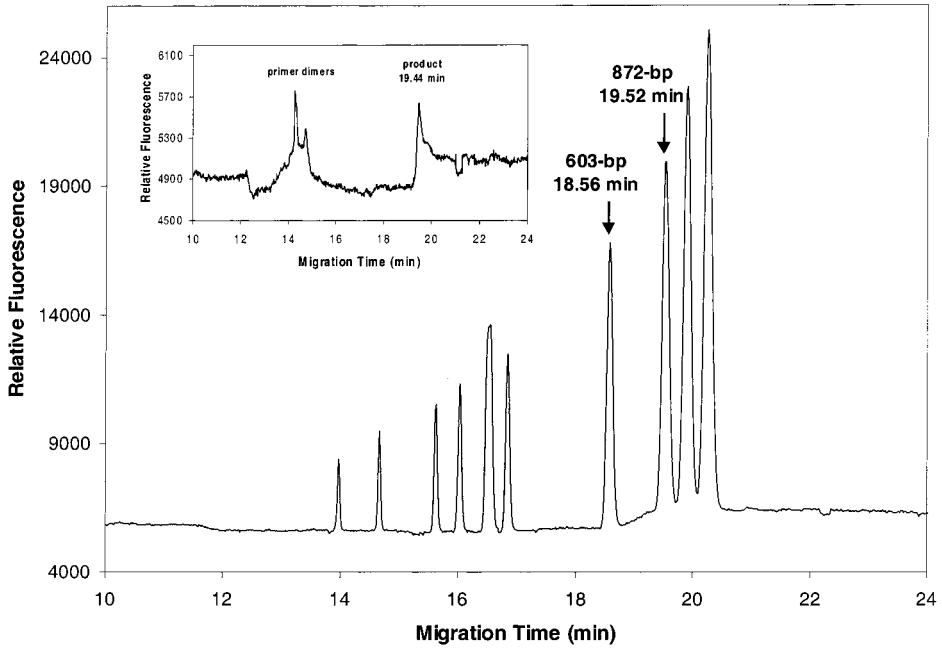


Figure 2. Electropherogram of 20 ng/mL ΦX174/*Hae*III DNA fragments. Inset shows an electropherogram of RT-PCR products using 838-bp positive control DNA as the template. RT-PCR was performed using a 25-amol template in 12.5 $\mu$L of RT-PCR solution. Sieving buffer: 1% HPMC-5, 0.5% PVP, and 6% mannitol, in TBE buffer containing 0.5 $\mu$g/mL ethidium bromide. Capillary, 40 cm $\times$ 50 $\mu$m i.d. (360 $\mu$m o.d.) with 25-cm effective length; electrokinetic injection for 5 s at −7.8 kV; electrophoresis voltage, −7.8 kV; excitation, 543-nm green helium−neon laser.

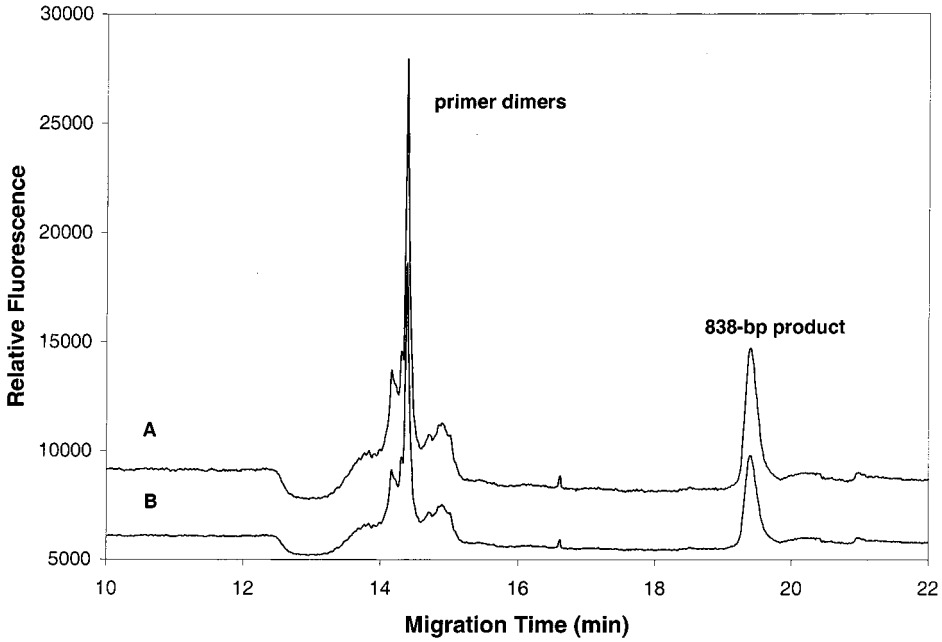


Figure 3. Electropherograms of RT-PCR products of single LNCaP cells: (A) freeze−thaw lysis; (B) SDS (0.05%) lysis. Conditions for CE-LIF were the same as in Figure 2.

than for freeze−thaw lysis. The difference in S/N between the two methods is due to variation in RT-PCR efficiency, rather than variation in CE-LIF. The RSD in S/N assessed with the 872-bp fragment of a ΦX174/*Hae*III DNA standard (20 ng/mL) was 5.3% ($n = 3$). Specifically, the reduction in S/N with detergent lysis is probably due to partial denaturing of the reaction enzymes by the SDS. Several nonionic detergents, including Tween 20 and Triton X-100, were also tested at concentrations ranging from 0.01 to 5%. However, complete lysis of the cells was not achieved using these detergents, even after waiting 5−10 min. For that reason, these detergents were not further examined in any RT-PCR experiments.

**Detection Sensitivity of cDNA Products.** To examine the sensitivity of our system, RT-PCR was performed with 25 amol ($\sim$1.5 $\times$ $10^7$ starting cDNA molecules) of the manufacturer-provided 838-bp DNA template in 12.5 $\mu$L of RT-PCR mixture. Reverse transcription did not occur with this standard (because it was already in the form of cDNA); however, a theoretical extrapolation can be made to estimate our limit of detection (LOD) in reference to a starting mRNA concentration. Assuming a

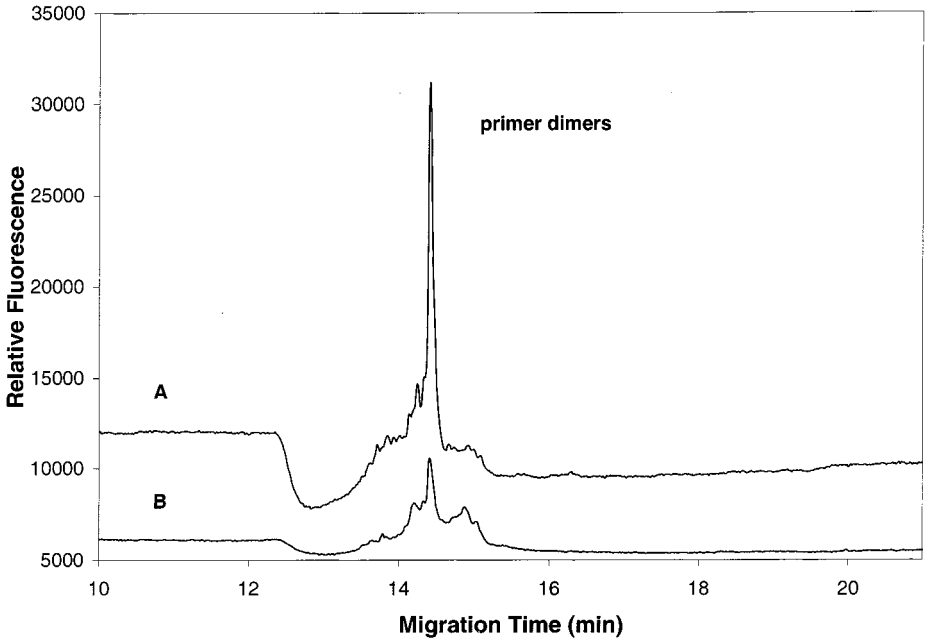


Figure 4. Electropherograms of RT-PCR control experiments. (A) Negative control: ~200 nL of sieving matrix was substituted for the cell during RT-PCR to test for the presence of exogenous RNA/DNA contamination. (B) Single-cell RT-PCR was performed in the absence of reverse transcriptase to test for interference of genomic DNA. Conditions for CE-LIF were the same as in Figure 2.

conservative 1:1 ratio of cDNA to mRNA, $\sim 1.5 \times 10^7$ starting mRNA molecules (for $\beta$-actin) would be present in 12.5 $\mu$L, which corresponds to ~1200 starting mRNA molecules/nL. A 2.4-nL injection of the amplified cDNA sample (i.e., following PCR) resulted in a product peak with a S/N ~ 18. Extrapolating linearly from a S/N = 18 to a S/N = 2, we achieve a theoretical LOD for our system of ~133 starting molecules/nL. Although PCR amplification of single-copy DNA molecules and subsequent detection with CE-LIF has been performed by others,[50−52] the addition of the RT step in a one-pot reaction mixture presents additional detection challenges. Highly efficient PCR (hence, sensitive detection) relies on an optimized $Mg^{2+}$ concentration. But unfortunately, a particular $Mg^{2+}$ concentration optimized for PCR efficiency can cause detrimental effects to RT efficiency, depending on cell type.[53] Hence, to preserve the integrity of the RT reaction at the single-cell level, our PCR efficiency is probably lower than that reported by others. It is important to note that detailed optimization studies have not been performed with our system as of this time. Therefore, we expect that our achievable LOD is probably much lower than what we are reporting here.

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## CONCLUSION

Capillary electrophoresis was used to monitor the expression of $\beta$-actin transcripts in single mammalian cells using RT-PCR. Using a single intact cell in conjunction with uncomplicated lysis methods simplifies RT-PCR by eliminating the need for complex RNA isolation techniques. CE offers many advantages over traditional slab gel electrophoresis for detection of single-cell RT-PCR products, including higher sensitivity and lower sample volume requirements. With further optimization, we should theoretically detect amplification products from only a few starting molecules, enabling the detection of transcripts originating from rare and low copy number genes. Future plans include optimization of RT-PCR conditions and the use of CE to quantitate $\beta$-actin transcripts from single cells.

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