

# An Improved Workflow for Sensitive qRT-PCR Gene Expression Studies of Single Cultured Cells

Judith E. Meis, Anu Khanna, Haiying Grunenwald, and Jim Pease

Epicentre (an Illumina® company), 726 Post Rd, Madison, WI 53713, USA

## Introduction

The variation in the gene expression profile of very small populations of cells provides a powerful insight into our understanding of developmental biology, e.g., how cells differentiate. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) offers a high-throughput, low-cost method to evaluate the expression of one to several genes (RNA transcripts). qRT-PCR, however, is problematic when using very small numbers of cells, for a variety of reasons that include: i) difficult and irreproducible purification of extremely small amounts of RNA; ii) low detection sensitivity, especially of low-abundance transcripts; iii) the very limited number of qRT-PCRs that can be performed; iv) the need to collect samples often; and v) the inability to archive samples for future analyses.

We describe an improved qRT-PCR template preparation workflow that greatly simplifies and improves qRT-PCR studies from very small populations of cultured cells, including from single cells. Our procedure eliminates the need to purify RNA, “preamplifies” the cellular mRNA by about 1,000-fold directly from a cell lysate, and reverse-transcribes the amplified RNA into cDNA that can be diluted for up to hundreds of sensitive qPCRs or archived for subsequent use.

## Methods

An overview of the qRT-PCR template preparation process is shown in Figure 1. Briefly, cultured cells were harvested, counted, diluted, and pelleted by standard laboratory procedures. Single-cell samples were obtained by trapping a single cell in a capillary (Wiretrol® II, Drummond Scientific). The pelleted cells were washed and resuspended in 3-10 µl of a commercial lysis solution (QuickExtract™ RNA Extraction Solution; Epicentre) and the cells lysed by vigorous vortex mixing or freeze-thaw. A 3-µl aliquot of the cell lysate was used for subsequent RNA amplification and cDNA synthesis. The cellular mRNA (poly(A) RNA) was amplified by approximately 1,000-fold directly in the cell lysate, using an improved linear T7 RNA polymerase-based *in vitro* transcription (IVT) procedure that is known to preserve the relative abundance of the RNA transcripts in a sample. The amplified RNA was treated with DNase I to remove the IVT template and the genomic DNA, and then purified using an RNA Clean & Concentrator™ column (Zymo Research). Finally, the amplified RNA was reverse-transcribed, using random primers, into cDNA in a final volume of 12-15 µl. The cDNA produced by the procedure can be diluted for qPCR or archived for future use. The entire cell lysis, RNA amplification, and cDNA synthesis procedure takes 6-8 hours.

This process can be used for one to 50 cultured cells, as well as cells harvested by laser-capture microdissection (LCM) techniques.

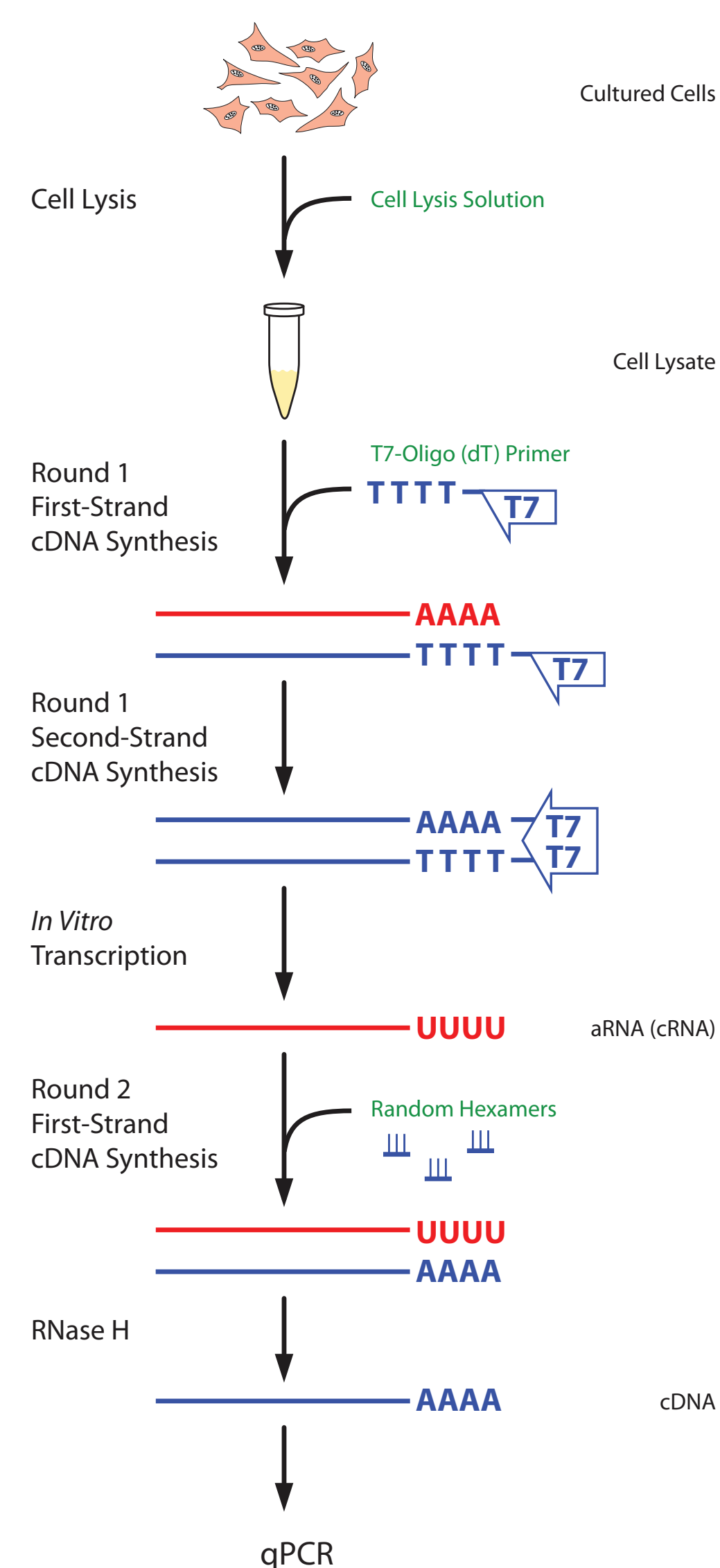


Figure 1. An overview of the RNA amplification and cDNA synthesis procedure.

## Results

### Greatly improved sensitivity of small-sample qRT-PCR

Amplified RNA was generated directly from the lysate of one or five cultured HeLa cells, converted to cDNA as described (Fig. 1), and designated as “Amplified cDNA”. A control without reverse transcriptase (designated “No RT”) was performed in order to detect residual contaminating genomic DNA. As a second control, a commercial “cDNA-from-cells”-type kit was used to make cDNA directly from cell lysates without the benefit of the RNA amplification step (designated “No Amplification”). The “No Amplification” control included a DNase I treatment to eliminate genomic DNA. Twenty-five microliter qPCRs (SYBR® Green detection) were performed using 1 µl of each cDNA preparation and a primer pair for the moderately expressed LDHA transcript.

As seen in Fig. 2A, our process greatly improved detection of the LDHA transcript from five cells ( $C_T = 19$ ) and from a single cell ( $C_T = 22$ ). The “No RT” and “No Amplification” controls produced only nonspecific signals, as determined by melting-curve analysis (Fig. 2B). These data demonstrate that the RNA amplification step can be performed directly in the cell lysate, without the need to purify RNA, and that the RNA amplification step enables sensitive and specific detection of transcripts from as little as one cell.

Further, the DNase treatment included in the post-IVT reaction reduces the amount of genomic DNA to virtually undetectable levels. In contrast, qPCR performed using cDNA produced without RNA amplification did not detect the desired transcript.

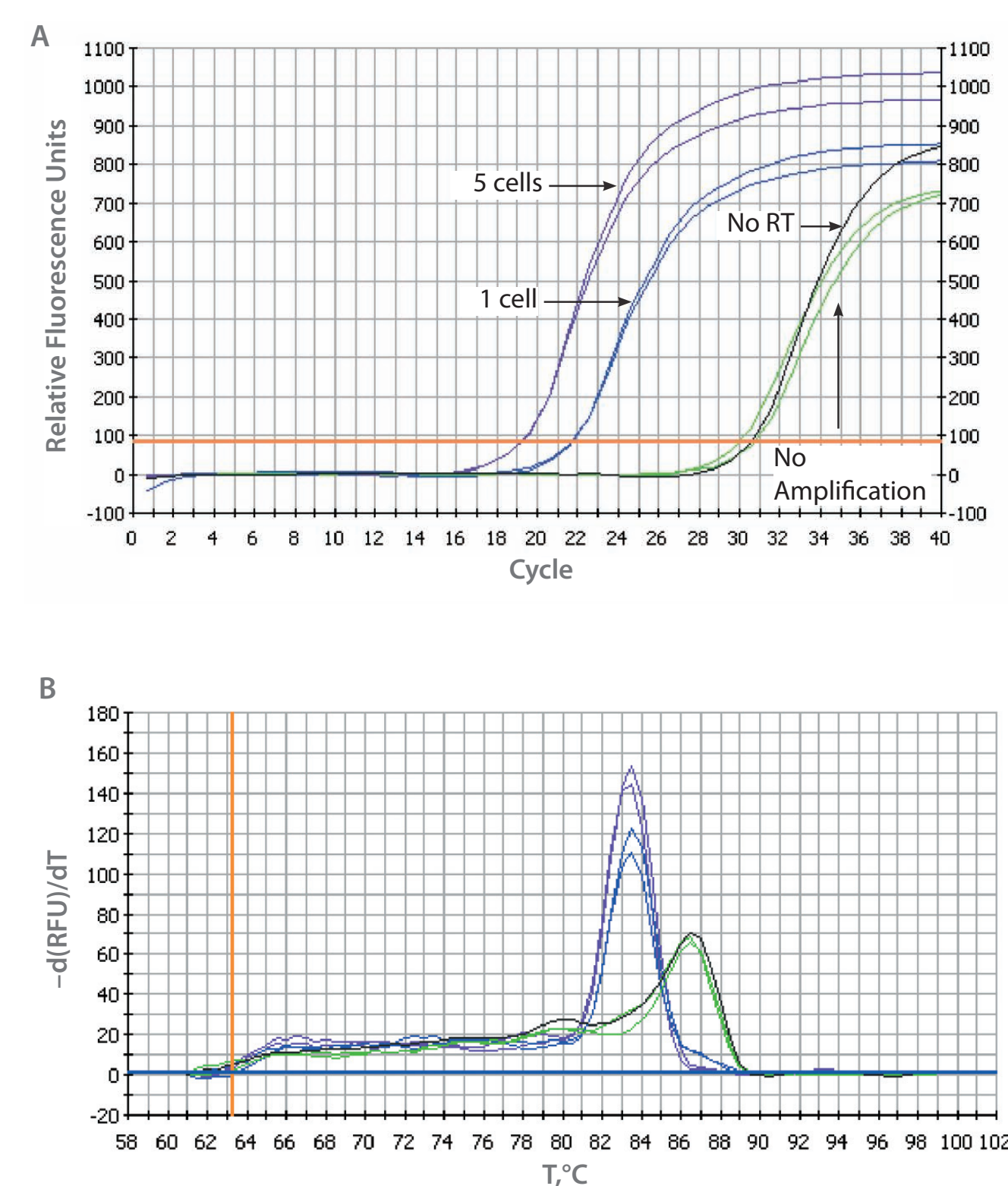


Figure 2. Sensitive and specific qRT-PCR from one cell. A) cDNA was produced directly from the lysate of five HeLa cells (purple) or one HeLa cell (blue) using the procedure described in Fig. 1. cDNA produced without reverse transcription (“No RT”; black) or without benefit of RNA amplification (“No Amplification”; green) were used as control reactions. The moderately abundant LDHA transcript was readily detected by qPCR (SYBR® Green) using cDNA produced from both the five-cell and the one-cell lysates, demonstrating that the RNA amplification greatly improves detection sensitivity while virtually eliminating genomic DNA contamination. B) Melt-curve analysis shows specific amplicons using cDNA produced by our procedure. In contrast, the qPCR of cDNA generated by the “No RT” and “No Amplification” controls generated nonspecific amplification products.

### Up to 100 qRT-PCRs from one cell

Amplified RNA was produced directly from the lysate of a single Normal Rat Kidney (NRK) cell and reverse-transcribed to cDNA by the procedure described in Fig. 1. One microliter of undiluted cDNA and cDNA diluted 1:10, 1:100, and 1:1,000 was used for qPCR (SYBR® Green detection) with a primer pair for the low-abundance PGBD transcript.

Based on the data of Fig. 3, qPCR performed using cDNA produced by our procedure and diluted 1:100 readily detects the low-abundance PGBD transcript. In each example, melt-curve analysis (data not shown) confirmed that the correct amplicon was produced. Thus, the RNA amplification and cDNA synthesis procedure described generates sufficient cDNA for 100 or more sensitive qPCRs directly from the lysate of one cell. The actual number of qRT-PCRs that can be obtained depends on the number of cells and the abundance of the transcript(s) of interest.

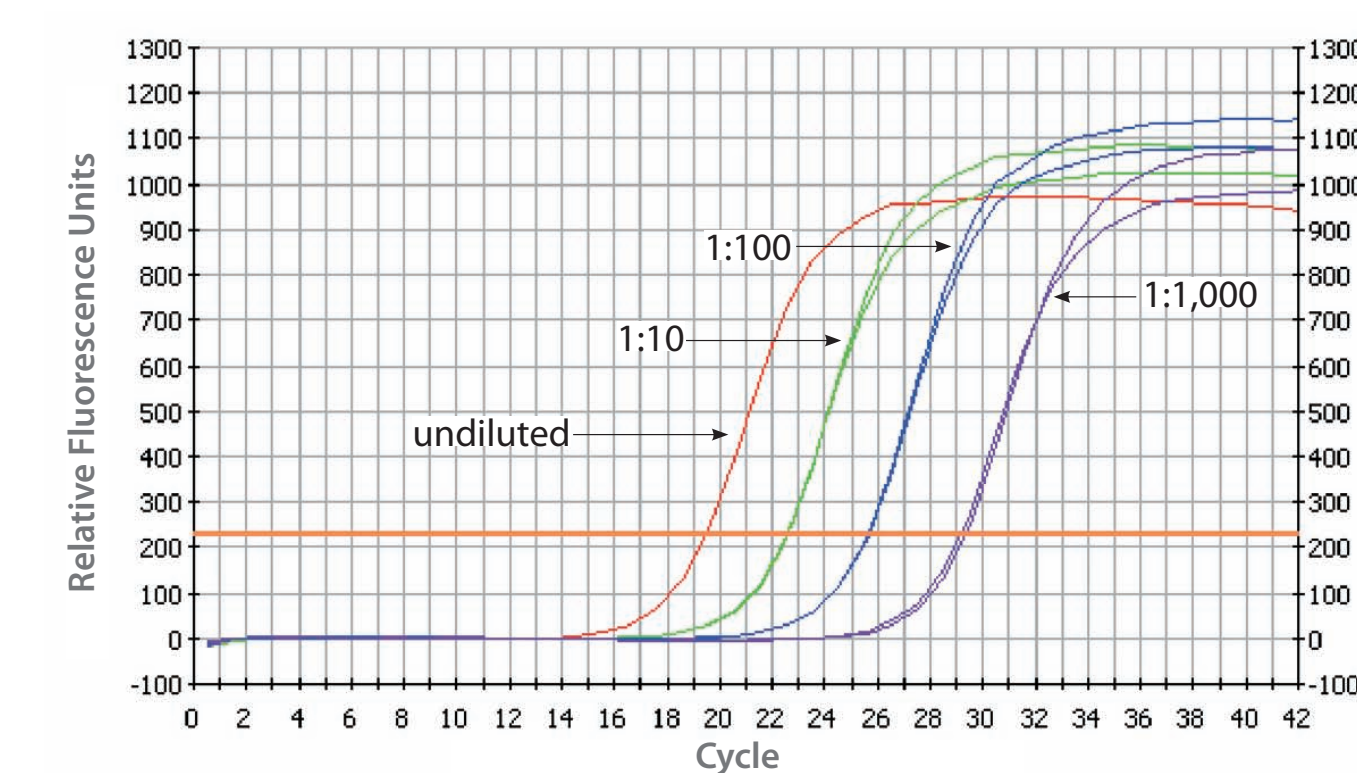


Figure 3. Up to 100 sensitive qRT-PCRs can be obtained from a single cell. cDNA was produced by the procedure described in Fig. 1. One microliter of the cDNA was used undiluted (red), diluted 1:10 (green), 1:100 (blue), and 1:1000 (purple) for qPCR (SYBR® Green detection) with a primer pair for the low-abundance PGBD transcript. This transcript was readily detected using cDNA diluted 1:100 ( $C_T \approx 25.5$ ). Melting-curve analysis (not shown) confirmed that the correct amplicons were produced at each cDNA dilution level.

## Conclusions

The small sample qRT-PCR template preparation procedure we describe eliminates many of the difficulties encountered in qRT-PCR studies using very small numbers of cells. Specifically:

- RNA amplification and cDNA synthesis can be performed directly in the lysate of as little as one cell, without the need to purify RNA and with no loss of RNA.
- Amplification of the mRNA directly from the cell lysate of as little as one cell produces sufficient amounts of cDNA for up to 100 or more sensitive qRT-PCRs.
- RNA amplification greatly improves detection of low-abundance transcripts.
- Sufficient cDNA is produced to archive for future use.
- The procedure eliminates genomic DNA, so primer pairs to any region of the transcript of interest can be used for qPCR.