*Labcyte White Paper*

# High Throughput RT-qPCR for Gene Expression Analysis

*Enabling technologies and practical considerations for implementation*

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## Overview

Simplified protocols have made RT-qPCR one of the most reliable techniques used in gene expression laboratories. It is the "gold standard" of gene expression analysis.

The current standard of accuracy for quantification of gene expression is reverse transcriptase quantitative PCR (RT-qPCR).[1] The method offers the highest level of precision, specificity, and sensitivity. Specialized instrumentation and kits manufactured by life science companies have simplified RT-qPCR protocols to the point that it is now the most straightforward and reliable investigative tool used in a multitude of laboratories performing gene expression. Researchers frequently use RT-qPCR to investigate cellular mRNA fluctuation in response to experimental conditions, such as treatment with small molecule compounds, protein therapeutic candidates, and foreign RNA.

While RT-qPCR is a valuable experimental technique, it is traditionally regarded as a low throughput method by comparison to other means of quantifying gene expression. Reasons for this include the number of steps required to produce resulting data, the amount of sample required, and the relatively high cost of reagents. As a result, higher throughput and more cost-effective assays are performed first. Once interesting experimental conditions are identified, RT-qPCR is used to validate lower-cost, higher throughput methods.

Recent improvements to RT-qPCR sample preparation reagents and protocols, combined with existing non-contact, nanoscale liquid handling technologies enable RT-qPCR assay automation, miniaturization, and optimization. Now, this "gold standard" of quantitative gene expression analysis can be used to improve productivity and efficiency by providing accurate gene expression data in earlier screening studies.

A new, cost-effective sample preparation method for RT-PCR incorporates a "one-step" cell lysis buffer which eliminates the need for mRNA isolation and purification.[2] This improved method, when com-

bined with nanoscale non-contact liquid handling[3] technology enables a high throughput automated workflow for gene expression.

## Acronym Disambiguation

Similarities between terms used to describe PCR-related technologies can be a source of confusion. Here, qPCR is an acronym for "quantitative polymerase chain reaction" and is used interchangeably with "real-time PCR." Both terms refer to quantification strategies based on the measurement of amplicon production during PCR as reflected by increasing levels of measured fluorescence emissions.

**The "RT" in RT-qPCR does not refer to "Real-Time," but rather to the ability to measure, quantitatively, RNA expression levels in samples of interest via Reverse Transcriptase.**

In the abbreviation "RT-qPCR," the "RT" refers to "reverse transcriptase," (not "real-time") an enzyme used to produce cDNA from mRNA.

## Polymerase Chain Reaction

The polymerase chain reaction (PCR) exponentially amplifies specific DNA fragments (amplicons) from a reaction mixture composed of DNA polymerase, the double-stranded DNA template bearing the sequence to be amplified, primers, free nucleotide triphosphates (dNTPs), and other buffer constituents required for the correct functioning of the DNA polymerase enzyme.[4] Each primer is a single strand DNA sequence designed and manufactured to direct PCR to specifically produce the desired amplicon only.

PCR was made practical as a laboratory DNA amplification technique by the introduction of thermostable DNA polymerases, which enable efficient amplification through repetitive thermal cycling.[5] During heating, the DNA strands dissociate to produce single-stranded DNA.

As the reaction cools, the primers anneal to complementary sequences on the template DNA and strand elongation occurs. As PCR proceeds, copies of the target DNA sequence accumulate exponentially with each heating and cooling cycle as the copies themselves become templates for amplification. PCR results were traditionally measured after the end of the amplicon production cycle with gel electrophoresis used to isolate amplified fragments by size. Gel electrophoresis was generally used for qualitative positive/negative confirmation, with limited quantitative value.

Gene expression is a function of messenger RNA (mRNA), which is produced in the cell as an intermediate step in protein production. Since DNA polymerases cannot amplify RNA, and RNA polymerases lack the proofreading capabilities of DNA polymerases, cellular mRNA samples must be converted back to DNA to run PCR. This is traditionally accomplished with a heat-stable enzyme, reverse transcriptase, that can be used to simplify the process of producing the requisite complementary DNA (cDNA) for PCR amplification.

**RT-qPCR specifically and precisely measures the amount of a specific target sequence, even in cases where only a single copy of the target sequence is present.**

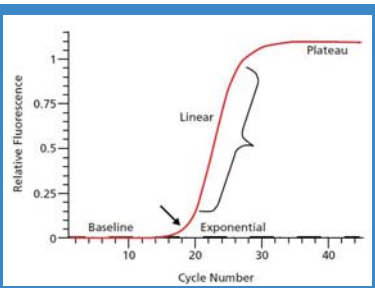
## Quantification by PCR

Unlike the end-point assays described previously, RT-qPCR experiments monitor amplicon production as the polymerase chain reaction occurs. This method specifically and precisely measures the amount of a specific target sequence in a reaction solution, even in cases where only a single copy of the target sequence is present in the first amplification cycle.

Amplicon production is monitored using a method such as fluorescence resonance energy transfer (FRET),[6] which enables quantitative measurements of multiple amplicons through the use of multiple fluo-

rophores with distinct emission wavelengths. Amplification and measurements occur simultaneously within a single instrument, which removes the requirement for additional steps and allows measurements to occur in real time.

In RT-qPCR, fluorescent signal correlates to the number of amplicons present in the sample. The number of amplification cycles required for fluorescence to reach a threshold over background (or crossing point) correlates to the initial number of copies of the target sequence present in the sample. A low but statistically meaningful threshold is chosen in order to take advantage of simple first-order reaction kinetics, which apply only insofar as the target sequence template is the limiting substrate and all other reaction components are present in excess.




**In RT-qPCR, the number of heating and cooling cycles required for the amount of detected fluorescence to reach a pre-determined threshold value (arrow) correlates to the initial number of copies of the target sequence present in the sample.**

## Gene Expression Analysis Methods

A powerful means of gaining insight into cell activity is to analyze the expression of one or more of its genes. Genes expressed by the cell produce proteins with specific purposes related to cell activity. Numerous factors regulate the expression of those genes that produce those proteins.

Clinical applications of gene expression analysis include:

- Monitoring disease progression
- Measuring cellular-level responses to experimental compounds
- Classification of certain strains and stages of cancer according to their gene expression signatures
- Determination of viral load
- Quantification of knock-out or knock-in gene delivery methods

There are many productive lines of inquiry into the analysis of gene expression. Many factors affect various cellular pathways and feedback mechanisms. However, transcription from DNA to mRNA is a required step in nearly every case. Therefore the quantification of mRNA encoded by genes of interest offers a useful index of their expression states.

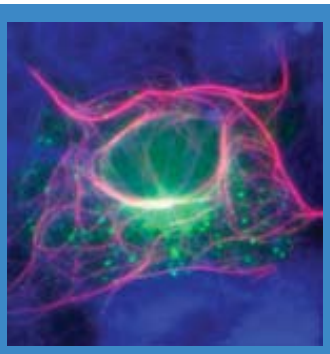
**The dynamic range of microarray systems is limited to a range between two and three orders of magnitude.**

The highly parallel format of a gene expression microarray enables observation of the effect of experimental variables on thousands of genes simultaneously. The dynamic range of microarray systems is limited to a range between two and three orders of magnitude, but the broad scope of simultaneous analyses makes this method useful for determining which genes, or groups of genes, are involved in producing the regulatory response to an experimental variable. Subsequent analysis by RT-qPCR can quantify reproducible gene expression regulation events, or determine whether certain data values may have arisen by chance or as the result of an experimental artifact. Microarray results may lead to a reporter gene assay screen, which focuses higher throughput screens to a particular gene of interest. Reporter gene assays[7] are currently one of the most popular methods of analyzing gene expression in higher throughput manners.

Reporter gene assays must be performed with an engineered cell line created by transfecting cells with cDNA encapsulated in a plasmid. The plasmid DNA bears a promoter or another regulatory element of a gene of interest, and a "reporter" gene, such as the gene for green fluorescent protein (GFP), which is usually independent of normal cellular functions. GFP production levels indicate that the cell line under analysis has encountered conditions under which the gene of interest is expressed, thus activating the promoter and causing the cell to produce GFP.

The number and diversity of transformed cell lines transfected with reporter gene fusion plasmids is limited. There is added complexity

because transfections may be transitory and rarely occur at 100% efficiency. To control for these possibilities, the plasmid often incorporates a second marker protein that is constitutively expressed—requiring a second set of measurements to track transfection efficiency as a control in order to ensure experimental accuracy. Designing and producing a new reporter cell line can become time consuming and unpredictable, and an abundance of intellectual property issues must be considered as part of the design process.



**Reporter gene assays, although attractive in concept, can be problematic to set up and manage.**

Regardless of origin, a reporter cell line is an artificial construct, and researchers must acknowledge that the observed regulatory responses of the plasmid-bearing transformed cell line may not accurately reflect gene regulation mechanisms as they apply to non-transformed cell lines, or to genes embedded within those cells. When the results of a reporter gene assay disclose the action of a regulatory phenomenon of interest, RT-qPCR on non-transformed cells may be subsequently relied upon to test related hypotheses and confirm experiments performed.

Several features of RT-qPCR contribute to its extraordinary value as a method of analyzing the regulation of gene expression:

- Unlike reporter gene assays, RT-qPCR can be used on native, non-transformed cell types, and RT-qPCR measures mRNA transcripts of the actual gene as it is expressed from within its natural environment, rather than transcripts from an artificial, plasmid-based construct.
- Assays are based on oligonucleotide sequences unique to the model cell line and gene product of interest, and are therefore extremely specific.
- RT-qPCR can amplify and detect one or more individual mRNA transcripts in a sample and can be used to analyze low expressing genes and very small samples, such as single cell-type populations.

- Results are reproducible and accurate over eight orders of dynamic range.

Quantification of gene expression based on mRNA traditionally required an intermediate experimental step to convert the mRNA to cDNA and to further purify it. This requirement added extra step complications to the process that limited sample throughput, and relegated RT-qPCR to a confirmatory technique. The conversion of a confirmatory low throughput method into a quantitative high throughput method requires advances in reagents, methods, and instrumentation—which must complement each other's function in a cost-effective workflow.

**Assay miniaturization offers significant economic advantages in the form of reduced reagent costs and reduced consumable costs. Leveraged over many thousands of assays, the benefits can be considerable.**

## Optimizing RT-qPCR for High Throughput

Features of high throughput RT-qPCR include:

- Precise, accurate quantification.
- Removal of the requirement to separately produce and purify cDNA.
- Assay miniaturization using 384- or 1536-well microplates, including reduction of assay scale to less than one microliter total reaction volumes.
- Acoustic, non-contact liquid handing for accurate nanoscale-volume dispensing of diverse-viscosity solutions without pipet tip consumption or cross contamination.
- Lower operational costs due to nanoscale reagent consumption and elimination of pipette tip use and disposal.
- Automated plate handling when "walk-away" time must be maximized.

High throughput RT-qPCR replaces other methods of quantification and moves PCR-based quantification into upstream screening. This has the benefit of saving time, and more importantly, of generating data that is more quantitative, more precise, and more sensitive than the methods replaced.

New sample preparation methods eliminate the requirement to produce and purify intermediate cDNA. To efficiently perform RT-qPCR in a high throughput manner, required reaction volumes also must be reduced. This moves the burden from reagents to equipment—liquid handlers must be accurate and precise at lower volumes to prevent transfer issues from interfering with the quantitative results of a small-scale assay. Non-contact acoustic fluid transfer is an approach to removing this experimental risk and enables higher throughput RT-qPCR workflows.

**It is no longer necessary to purify RNA samples as part of the RT-qPCR process. New "one-step" reagents allow you to go directly from cell lysates to the RT-qPCR reaction.**

In conventional, standard throughput RT-qPCR, the principal bottleneck is the requirement for purified mRNA starting material. For a typical experiment performed on cultured cells, this meant that extensive bead-based or column-based protocols were required. Purification of mRNA involves protocols with multiple wash steps to produce mRNA free of contaminating nucleases, genomic DNA, reverse-transcriptase inhibitors and PCR inhibitors. The production of cDNA would often involve another multi-step protocol with an additional set of reagents. By contrast, new "one-step" buffer systems eliminate the purification bottleneck by allowing RT-qPCR quantification of mRNA directly from cell lysates. While this feature is of certain convenience to gene-expression researchers performing low throughput benchtop RT-qPCR, its principal benefit to all researchers is compatibility with laboratory automation systems. "One step" sample preparation, available as the RealTime ready Cell Lysis Kit from Roche, for example, makes RT-qPCR faster, simpler, and easier to integrate into an automated workflow.

The simplification of experimental steps required to produce RT-

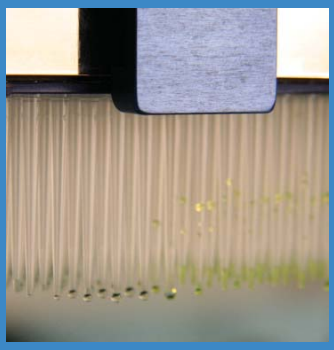
qPCR samples is an important factor in making it a feasible higher throughput technique. There are several other factors which enable better throughput:

- RT-qPCR is a technique that performs measurements as the sequence of interest is amplified. Thus, a single instrument will require between 30 and 90 minutes to perform an assay.

- Many RT-qPCR instruments use lower scale formats, such as 96-well formats. To run at a higher throughput scale, multiple instruments would be required. The physical space and cost required for multiple detection instruments reduces the ability to automate in a high-throughput manner. Newer models and instrumentation for RT-qPCR allow formats in 384-well or 1536-well formats, thus allowing up to 16 times the number of samples in a set. This reduces the number of RT-qPCR instruments required to scale the method into higher throughput formats.

- Performing an experiment at high throughput, by necessity, removes manual liquid transfer steps to transform a benchtop, low throughput, manual technique into a high throughput, automated technique. An increase of well density from 96-wells to 1536-wells, the reaction volume may decrease volumes by 95%. While this has the added benefit of greatly reducing reagent costs, automated liquid handling at low volumes remains a challenge.

- For automated liquid handling, the cost of disposable tips and the performance at low volumes may be limiting factors in the adoption or miniaturization of valuable experimental techniques. Disposable tips are used in manual methods to eliminate the risk of sample carryover. Sensitive techniques such as RT-qPCR add some degree of experimental risk as the technique will detect small but meaningful changes in ampli-



**The elimination of pipet tips with acoustic dispensers can significantly reduce assay costs.**

con levels, even down to single copies. A reduction of scale to 1536-well formats, for example, adds additional challenges to precise and accurate liquid transfer methodologies, since most tip-based methods exhibit less accurate and precise transfer as a function of reduced transfer volumes.

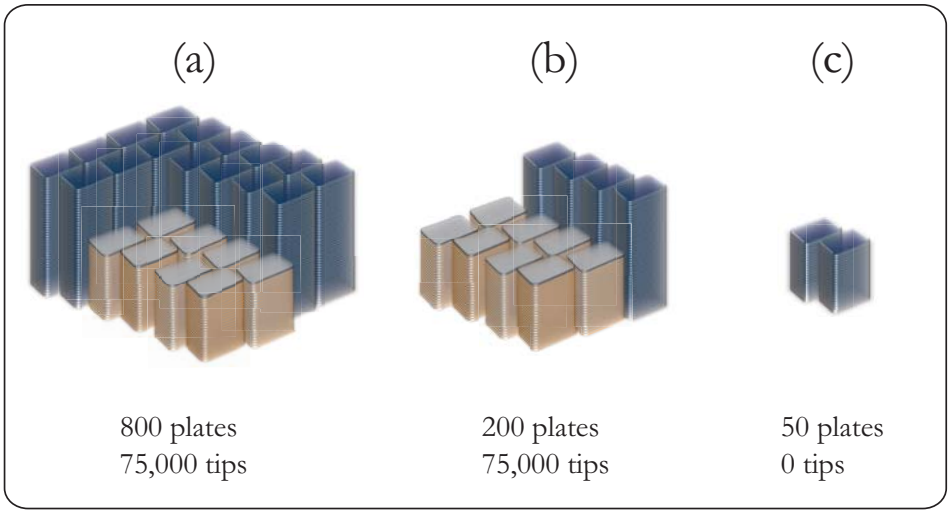
- Acoustic liquid transfer methods have been utilized as a technique to overcome the challenges inherent in transforming a low throughput method into a small-scale, automated high throughput screening technique. Focused acoustic energy pulses eject fluid droplets of a discrete size from a source into an inverted destination; fluid surface tension properties allow the transferred fluid to remain in place. The Labcyte Echo® 500 series of liquid handlers have been utilized to transfer RT-qPCR reagents, cDNA and cell lysate samples into 384-well and 1536-well formats. Since the instruments use non-contact transfer there are no associated tip costs, and no risk of cross-contamination. The Echo liquid handlers transfer drops of fluid at 2.5 nL increments; larger fluid volumes are transferred by rapidly repeating the transfer steps several hundred times per second.

- Many liquid handlers require separate calibrations to transfer reagents of different viscosities—unfortunately these differences are not always known or apparent to researchers *a priori*. High throughput screens on cell-based assays may produce results with varying levels of cell density and therefore different levels of cell lysate viscosity. For many automated liquid handlers this could cause inconsistent results simply because a common calibration would be required to transfer samples of different fluid properties. While nearly all liquid handlers require some level of calibration, acoustic transfer methods as implemented by Labcyte utilize real-time, dynamic fluid analysis methodologies that adjust acoustic pulse power levels on a sample-by-sample basis. This ensures that samples with small but significant differences in cell lysate viscosity can still be

consistently transferred, and removes the requirement for the researcher to create multiple calibrations for the smaller volume fluid transfer steps.

## Avoiding Cross-Contamination in High-Throughput RT-qPCR

With conventional liquid handing, disposable tips are the only way to avoid cross-contamination—but they have a cost that adds up quickly. Non-contact dispensing with a Labcyte 500 series Echo platform eliminates the risk of cross-contamination without consuming tips. The figure shows a conservative estimate of tip usage for 40,000 PCR experiments.




*Estimated plate and tip usage for a 40,000 RT-qPCR sample setup. For more traditional 96-well qPCR setups (a) approximately 800 plates and a minimum of 75,000 disposable tips would be required. 400 plates are used to grow cell cultures and 400 plates for the actual qPCR setup. Higher density 384-well formats (b) will reduce the plate requirements but will not reduce the required number of disposable tips. Increasing the density even further to 1536-well formats (c) and using the Echo liquid handler to transfer reagents and cell lysates decreases plate requirements to 50 plates and eliminates the disposable tip requirements.*

# The High Throughput RT-qPCR Workflow

The high throughput RT-qPCR workflow consists of the following steps:

| Workflow Step | Details |
|---|---|
| Cell Seeding | Cells are transferred into an Echo® qualified tissue culture treated microplate and grown overnight. |
| Treatment | Cells are screened against compounds that may induce gene expression changes. |
| One-Step Cell Lysis Buffer Addition | Cell culture media is removed and cells are lysed. One-step lysis buffer removes the requirement to separately produce and purify mRNA and cDNA. |
| qPCR Setup | Reagents and cell lysate are transferred into a PCR microplate. |
| Plate Sealing and Centrifugation | PCR microplates are sealed and centrifuged in preparation for analysis. |
| qPCR analysis | Thermal cycle to quantitatively determine expression of genes of interest. |

When scientists are readily available at required plate transfer intervals, or the high throughput RT-qPCR workflow has been automated by a platform such as the Labcyte Access™ workstation, assay throughput is limited only by the speed of the instrument used for thermal cycling and fluorescence quantification. The Roche LightCycler® 480 or 1536 systems require approximately 45 minutes to analyze one PCR microplate. In a typical eight-hour work shift there is sufficient time to prepare 28 PCR plates, enough to keep two LightCycler® 480 or 1536 systems busy overnight. With the option to integrate multiple Light-

Cycler® systems, the Access workstation can achieve a daily sample throughput of 10,752 reactions in 384-well plates, or 43,008 reactions in 1536-well plates. To scale the process with more automation, the integration of incubators and multiple plate handling devices as offered with the Labcyte POD™ automation platform, adds cell plating, storage and maintenance to automated high throughput RT-qPCR.

## Summary

RT-qPCR is the recognized "gold standard" of transcript quantification, but its utilization has been limited to the validation of small data sets produced by higher throughput methods. While this approach has been used productively, it requires an inefficient two-step approach.

By comparison to available alternatives, the high-throughput RT-qPCR assay described above is efficient, economical, and broadly applicable. Non-contact acoustic liquid handling both improves performance and enables better results, even in lower volumes and increased sample density. High throughput RT-qPCR has been welcomed by researchers requiring sensitivity and gold-standard accuracy with a high throughput approach to analyzing transcript abundance. As a result of advances in reagent development and laboratory automation, any existing benchtop RT-qPCR assay can now be introduced into a high throughput workflow capable of producing tens of thousands of data points per day.

## References

1. Canales, R.D., et al. 2006. Evaluation of DNA microarray results with quantitative gene expression platforms. Nat. Biotechnol. 24:1115-1122.

2. Roche Diagnostics GmbH. Technical Note: Fast One-Step Cell Lysis; RealTime ready Cell Lysis Kit. 2010.

3. Ellson, R., Mutz, M., Browning, B., Lee, L., Miller, M.F., Papen, R. 2003. Transfer of low nanoliter volumes between microwell plates using focused acoustics – automation considerations. J. Am. Laboratory Assoc. 8:29–34.

4. Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim, N. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350-1354.

5. Lawyer, F.C., Stoffel, S., Saiki, R.K., Chang, S-Y., Landre, P.A., Abramson, R.D., and Gelfand, D.H. 1993. High-level expression, purification, and enzymatic characterization of full-length Thermus aquaticus DNA polymerase and a truncated form deficient in 5' to 3' exonuclease activity. Genome Res. 2:275-287.

6. Bernard, P.S., Lay, M.J., and Wittwer, C.T. 1998. Integrated amplification and detection of the C677T point mutation in the methylenetetrahydrofolate reductase gene by fluorescence resonance energy transfer and probe melting curves. Anal. Biochem. 255:101-107.

7. Naylor, L.S. 1999. Reporter gene technology: the future looks bright. Biochem. Pharmacol. 58:749-757.