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Tech Note

The Changing Face of HTS

Automating and Miniaturizing Gene Expression Assays via High-Throughput RT-qPCR

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Luciferase (firefly) reporter gene assays have been the industry standard in high-throughput screening (HTS) for more than a decade. They are still widely used in biomedical and pharmaceutical research. Advantages of using a reporter gene assay for gene-expression analysis have been the speed of the reaction time, lower price point per reaction, and the absence of luciferase activity in most cell types, which prevents ambiguity and crosstalk.

There is, however, a growing consensus among scientists that reporter gene assays are not ideal for performing gene-expression analysis, for the following reasons:

1. *Lengthy procedures:* A time-consuming, multistep process may separate the identification of the target gene of interest and the acquisition of the endpoint assay result. A typical hindrance is the need to generate stable or transiently transfected constructs in cell lines.
2. *Limited biological relevance:* Reporter gene assays limit researchers to using immortalized cell systems and are unable to measure endogenous gene expression. Because reporter gene assays cannot be used on native, non-transformed cell types, they cannot measure the mRNA transcripts of the actual gene as it is expressed within its natural promoters and chromatin context; rather, it can only measure the transcripts from an artificial plasmid-based construct, which limits the biological relevance of the data.
3. *False hits:* There is a body of published data highlighting the issue of false hits with reporter gene assays, such as those caused by luciferase inhibition and leaky expression of reporter.

Evolution of HTS

Reverse transcriptase quantitative PCR (RT-qPCR) is the industry standard of accuracy and sensitivity for quantification of gene expression. RT-qPCR is often used to investigate cellular mRNA fluctuation in response to experimental conditions, such as treatment with small molecule compounds, protein therapeutic candidates, and foreign RNA.

RT-qPCR, however, has been traditionally regarded as a low- to medium-throughput method due to A) the number of manual steps required to isolate nucleic acids, B) expensive large-volume reactions associated with RT-qPCR, and C) the inability to automate the process from sample to result. Accordingly,

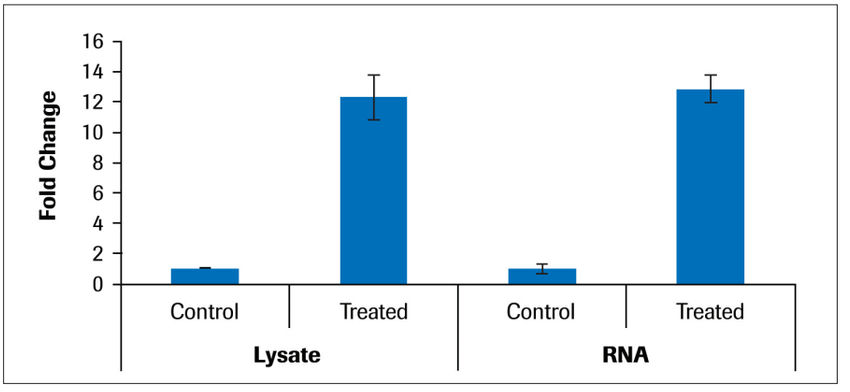

Figure 1. Comparison of RT-qPCR results obtained by using either lysate generated with the RealTime ready Cell Lysis Kit or purified RNA as template. Reaction volume: 0.5 µL. Reaction components: RealTime ready RNA Virus Master, 500 nM primer, 400 nM probe, 10% input template, *n* = 6. Data on file.

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many screening groups employ reporter gene assays as their primary screening method and, subsequently, utilize RT-qPCR to validate the hits in a secondary screen.

Recent improvements to RT-qPCR sample-preparation reagents and protocols, combined with existing noncontact, nanoscale liquid-handling technologies, have enabled RT-qPCR assay automation and miniaturization. The new single-step cell lysis buffer from **Roche**, RealTime ready Cell Lysis Buffer, eliminates the need for mRNA isolation and purification, and the corresponding Roche master mix enables direct use of the lysate in an RT-qPCR.

Data show that quantification cycles (Cq values) from RT-qPCR performed with lysate and those from purified RNA are highly correlated (*Figures 1 & 2*). To ascertain the Cq values, 30,000 HeLa cell equivalents/well were used for RNA preparation, and RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit. Real-time PCR amplification was performed on a real-time PCR instrument (96-well format, 20 µL reaction) using the LightCycler® 480 Probes Master and RealTime ready Human Reference Gene Panel.

The total process, from cells to results, takes as little as 90 minutes and avoids the issues of biological relevance that plague reporter gene assays. Performed on the Roche LightCycler 1536 instrument, this approach facilitates the processing of as many as 16 1,536-well plates per 24 hours. The entire workflow is completely automatable, with no manual intervention from cells to results.

Commenting on these new developments, Lynn Rasmussen, high-throughput screening center supervisor at Southern Research Institute, said the following: "All assay platforms have limitations, and reporter assays using luciferase, GFP, etc. have their own problems. There are cases when it is very difficult to engineer a reporter into a system, for example: RNA viruses with stringent packaging requirements, such as influenza. qPCR can be used in place of reporters to look at native messages in context, to confirm actives from reporter assays, and to monitor virus production in antiviral assays. The limitations of qPCR for HTS applications have been throughput and cost. The introduction of 1,536-well qPCR has addressed these limitations."

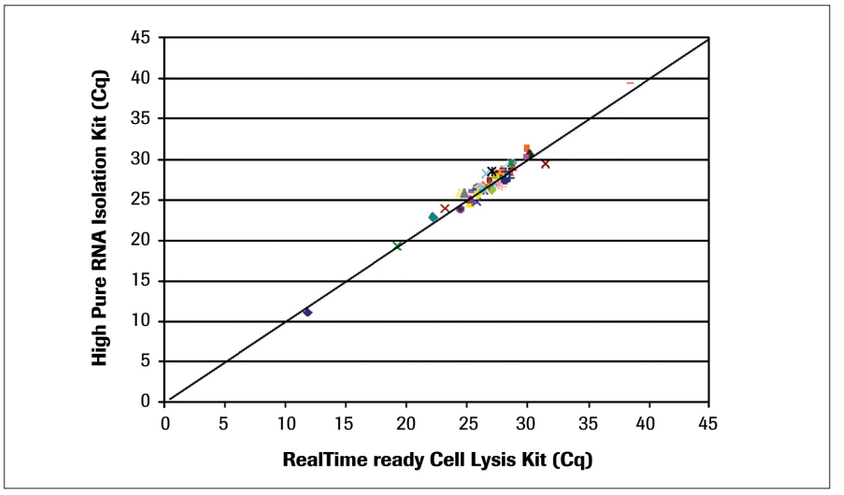

Figure 2. Real-time PCR results using lysate generated with the RealTime ready Cell Lysis Kit or RNA purified with the column-based High Pure RNA Isolation Kit. Analysis shows accurate, reproducible results for both RNA isolation techniques, resulting in high correlation of quantification cycles (Cq values) for the 19 different reference genes tested.

RT-qPCR in Screening

The combination of the LightCycler 1536 instrument, plate- and liquid-handler instruments, plate sealer, and centrifuge can be automated and controlled by scheduling software. The LightCycler 1536 instrument is a completely open platform capable of interfacing with a wide variety of third-party automation vendors. The fully automated high-throughput RT-qPCR workflow, using cell lysate as a template, consists of the following steps:

1. *Cell seeding:* Cells are transferred into a 384- or 1,536-well tissue culture treated microplate and grown overnight.
2. *Treatment:* Cells are screened against compounds that may modulate gene-expression changes.
3. *One-step cell lysis buffer addition:* Cell culture media is removed and cells are lysed with the RealTime ready Cell Lysis Buffer (5 minutes). The one-step protocol eliminates the need to separately produce and purify mRNA and cDNA.
4. *qPCR setup:* Reagents and cell lysate are transferred into a PCR microplate.
5. *Plate sealing and centrifugation:* PCR microplates are sealed and centrifuged in preparation for analysis.
6. *qPCR analysis:* Thermal cycling (e.g., on the LightCycler 1536 system) quantitatively measures expression of genes of interest.

Conclusion

The adoption of high-throughput RT-qPCR offers distinct advantages for gene -expression profiling in the context of compound screening, especially at the early stages of drug discovery. The miniaturized, high-throughput format afforded by the LightCycler 1536 instrument now allows for the application of this technique at stages earlier than post-screening validation steps, and in a completely automated workflow. The use of RT-qPCR in HTS increases the sensitivity and biological relevance of hits, giving researchers confidence that no potential compounds were missed. GEN