High-throughput qPCR and RT-qPCR Workflows Enabled by Beckman Coulter Echo Acoustic Liquid Handling and NEB® Luna® Reagents

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Introduction

Quantitative PCR (qPCR) is a simple but powerful method for the detection and quantitation of target nucleic acid sequences. The technique employs a real-time PCR instrument to measure the amount of a DNA target present at each cycle of a PCR, as determined via the fluorescence of either a DNA intercalating dye (e.g., SYBR® Green I) or a fluorescently-labeled probe (e.g., TaqMan). The cycle at which a threshold fluorescence (C_q value) is achieved during exponential PCR amplification is then used to calculate the starting quantity of DNA, by comparison either to a control (relative quantitation) or to standards of known quantity (absolute quantitation). For RNA quantitation, DNA amplification by PCR is preceded by a reverse transcription (RT) step to generate cDNA from RNA, carried out either separately (two-step RT-qPCR) or as an upfront step in the same reaction (one-step RT-qPCR).

The use of qPCR and RT-qPCR has become prevalent for a wide range of applications, including gene expression analysis, SNP identification, genotyping, contamination screening, and molecular diagnostics for cancer and infectious disease. For many of these applications, the ability to conduct qPCR experiments in high-throughput formats can present a tremendous advantage: gene expression studies can be expanded to include more targets, multiple controls, and additional replicates; screens can be conducted at larger scale; and diagnostic tests can accommodate more patient samples. 384-well real-time PCR instruments are readily available, improving throughput over standard 96-well platforms; however, manual reaction setup in this format can be tedious and challenging, with a higher potential for pipetting mistakes and increased variability from manually pipetting low volumes. Automated liquid handling can address these challenges, reducing error and improving efficiency to remove manual setup as a workflow bottleneck.

TABLE 1:
Echo-compatible Luna reagents and recommended liquid class settings

		ECHO-QUALIFIED PLATE TYPE		
REAGENT	NEB PRODUCT or COMPONENT #	384PP 2.0	384LDV or 384LDV PLUS	RESERVOIR
Luna qPCR reagents (for DNA templates)				
Luna qPCR Master Mix	M3003	GPSB	GP	GPSB
Luna qPCR Probe Master Mix	M3004	GPSB	GP	GPSB
Luna One-Step RT-qPCR reagents (for RNA or cell lysate templates)				
Luna Universal One-Step Reaction Mix	M3005	GPSB	GP	GPSB
Luna Universal Probe One-Step Reaction Mix	M3006	GPSB	GP	GPSB
Luna WarmStart® RT Enzyme Mix (20X)	M3002	GPSB	GP	GPSB
Other reaction components				
Luna Cell Ready Lysates	E3032/E3030/E3031	GPSB	GP	GPSB
DNA or RNA templates (in water or TE)	–	BP	GP	BP
Primers	–	BP	GP	BP

In this study, we show that NEB Luna qPCR reagents offer strong performance in high-throughput qPCR applications and are readily compatible with automated reaction setup, here using the Beckman Echo 525 acoustic liquid handler, a modern platform for tipless small-volume dispensing that is designed for use in biochemistry and genomics applications. Echo-mediated liquid transfer is achieved via Acoustic Droplet Ejection (ADE), which uses focused ultrasonic energy to eject small droplets of a defined volume (25 nanoliters for the Echo 525) from a source well into a destination well. This allows rapid, precise liquid transfer at nanoliter to microliter scales while avoiding tip-based cross-contamination and reducing plastic waste. The Echo 525 can assemble a full 384-well plate of qPCR reactions in less than 10 minutes, and is thus well-suited for high-throughput qPCR reaction setup. All Luna reagents are compatible with Echo-mediated acoustic transfer, and maintain sensitive detection and linear quantitation in 384-well format at reduced reaction volumes. In addition, using the Luna Cell Ready One-Step RT-qPCR Kit, we present a convenient workflow for automated direct RT-qPCR analysis of cell samples, with cell culture and lysis in Echo-qualified source plates providing transfer-ready template lysates for ADE-mediated reaction assembly and high-throughput RT-qPCR analysis.

Results

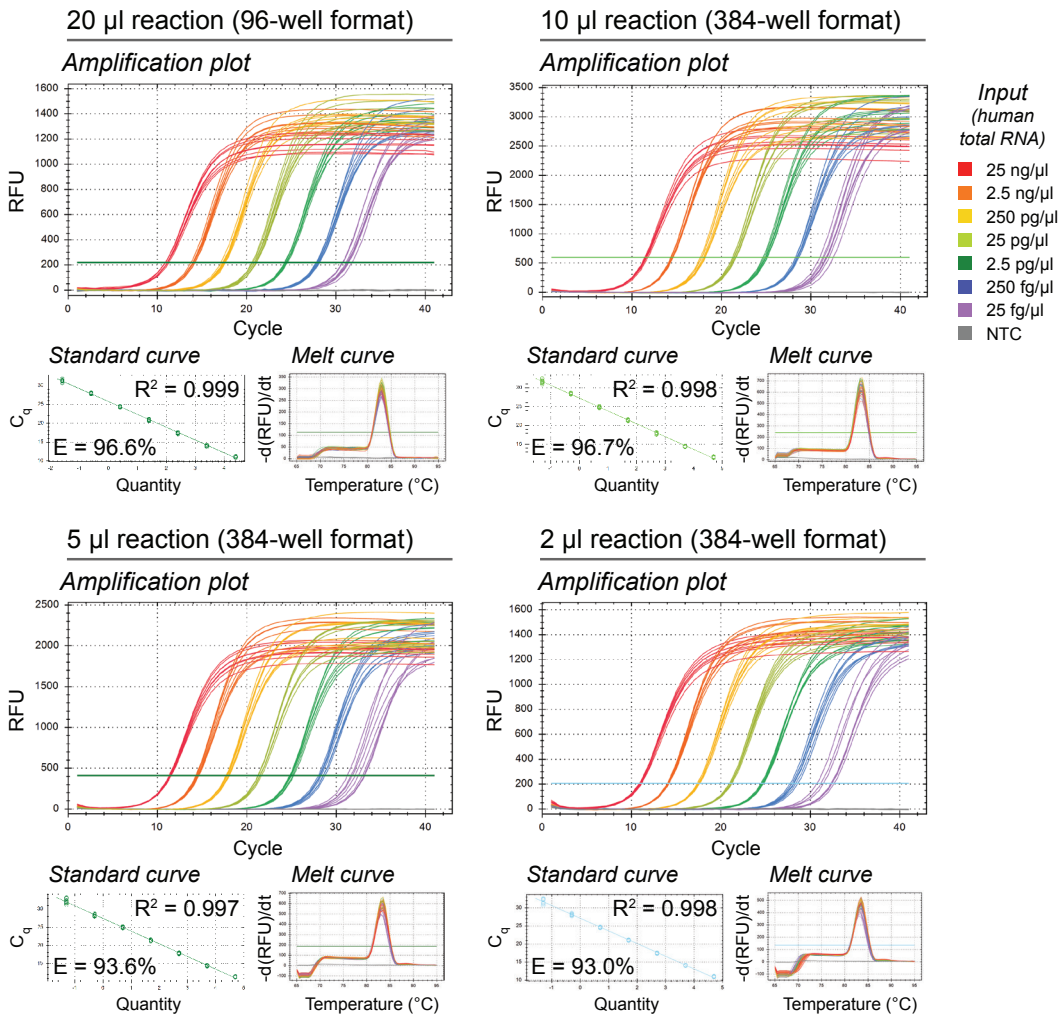
Echo Compatibility and Reaction Miniaturization

Using an Echo 525 Liquid Handler, we first verified compatibility of Luna reagents with acoustic liquid transfer. Luna kit reagents were precisely and reproducibly transferred to qPCR plates from a variety of Echo-qualified source plates. This was true for all Luna products evaluated, as summarized in Table 1 (Appendix).

To assess Luna reagent performance using Echo-mediated reaction setup, we examined sensitivity,

FIGURE 1:
Luna reagents are compatible with acoustic liquid transfer and reaction miniaturization

The Beckman Echo 525 Acoustic Liquid Handler was used to assemble Luna Universal One-Step RT-qPCR reactions at 10 µl, 5 µl and 2 µl volumes in a 384-well format, targeting human GAPDH for quantitation over a 7-log range of input template concentrations (25 ng/µl – 25 fg/µl Jurkat total RNA) with 8 replicates at each concentration. Linear quantitation and low-input detection were maintained in miniaturized reactions.



linearity, and reproducibility of quantitation for a well-characterized qPCR target, human GAPDH. A standard reaction volume of 10 µl is recommended for manual setup of Luna qPCR in 384-well format, in part to avoid the higher variability and potential for error that can be associated with small manual pipetting volumes (e.g., < 1 µl). Since the Echo liquid handler can dispense sub-microliter volumes with high precision, use of the Echo for setup also allowed us to test performance at lower reaction volumes of 5 µl and 2 µl (Figure 1). At all volumes, linear quantitation was observed over a 7-log input range (25 ng/µl to 25 fg/µl total RNA; efficiency ≥ 93% and $R^2 \geq 0.997$), with consistent detection at low input, and precise C_q values across replicates (n = 8, CV ≤ 2.3% at 25 fg/µl and ≤ 1% at higher inputs). This is consistent with Luna reagent performance at standard reaction volumes (e.g., 20 µl in 96-well format), demonstrating that robust performance can be maintained at lower reaction volumes and following ADE-based reaction assembly. When considering volume requirements for specific workflows, it should be noted that many real-time instruments recommend a minimum reaction volume of 5 µl, and lower volumes may negatively impact performance on certain platforms (e.g., due to evaporation and/or incompatibility with instrument configuration). For most applications, a reaction volume of 5 µl is therefore recommended for Echo-mediated setup; an example reaction setup is shown in Table 2 (Appendix).

TABLE 2:
5 µl reaction setup example:
Luna Universal Probe One-Step RT-qPCR Kit (NEB #E3006)

COMPONENT	5 µl REACTION	FINAL CONCENTRATION
Luna Universal Probe One-Step Reaction Mix (2X)*	2.5 µl	1X
Luna WarmStart RT Enzyme Mix (20X)*	0.25 µl	1X
Forward Primer (10 µM)	0.2 µl	0.4 µM
Reverse Primer (10 µM)	0.2 µl	0.4 µM
Probe (10 µM)	0.1 µl	0.2 µM
Template RNA		< 250 ng (total RNA)**
Nuclease-free Water	to 5 µl	

* Preparation of an assay mix (One-Step Reaction Mix + Enzyme Mix) is recommended to assure complete mixing.
** See usage notes in manual for additional guidelines on primer/probe design and template preparation/concentration.

FIGURE 2:
Evaluation of sensitivity and linearity for absolute quantitation of DNA and RNA over a broad input range

Templates of defined copy number were used to evaluate quantitation accuracy and detection sensitivity for Luna qPCR and RT-qPCR following Echo-mediated reaction assembly (4 µl each). Templates were evaluated over an 8-log input range (10^8 down to 10^1 copies, with 3, 6 or 9 reactions at each input). **A.** Quantitation of plasmid DNA bearing an AAV2 viral gene target using the Luna Universal qPCR Master Mix. **B.** Quantitation of ERCC00130 from ERCC (External RNA Controls Consortium) mix1 RNA using the Luna Universal One-Step RT-qPCR Kit. Linear quantitation (Efficiency ≅ 100%) was observed for both DNA and RNA targets, as well as sensitive detection at low input (6/6 replicates for AAV2 DNA target at 10 copies per reaction, 9/9 replicates for ERCC RNA target at 10 copies per reaction). 1 ng/µl total human RNA was used as carrier for ERCC template dilutions, and improved detection efficiency and replicate consistency at very low input (10 copies per reaction; 9/9 replicates detected and CV = 1.4% with carrier, 6/9 replicates detected and CV = 2.2% without carrier) (data not shown).

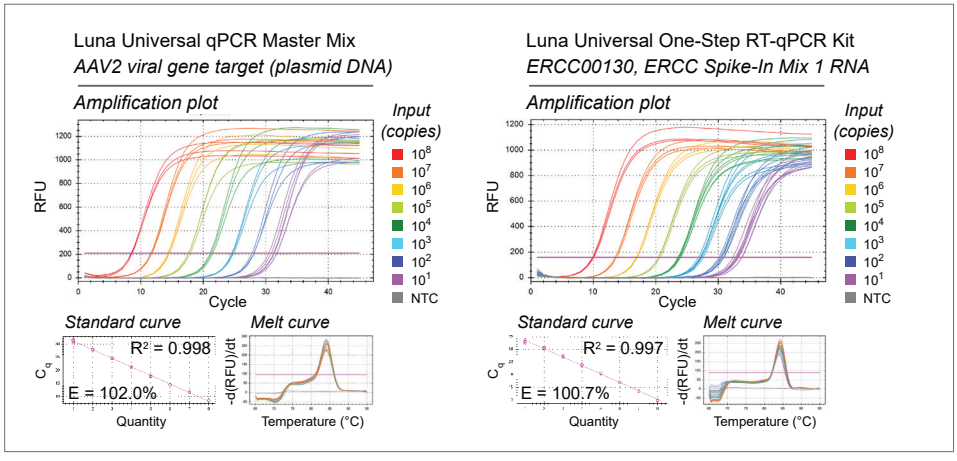
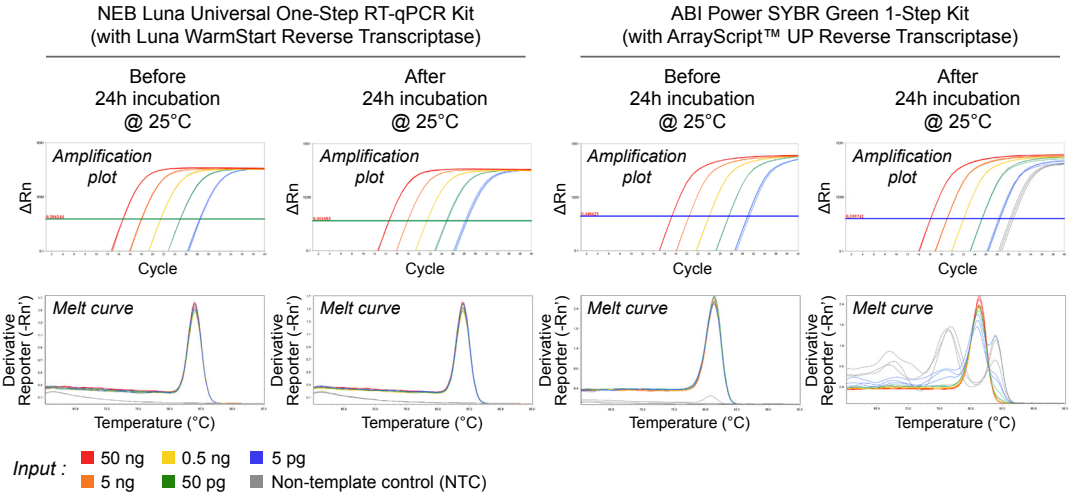


FIGURE 3:
Dual activity control with WarmStart Luna RT and Hot Start *Taq* prevents spurious amplification during room-temperature setup and wait steps

RT-qPCR targeting human ribosomal protein L32 was performed before and after a 24-hour incubation at room temperature, with triplicate reactions for a 5-log range of input human (Jurkat) total RNA and a non-template control. The Luna Universal One-Step RT-qPCR Kit featuring Luna WarmStart Reverse Transcriptase exhibited robust performance and no detectible non-template amplification, either with or without a 24 hour 25°C pre-incubation, while the ABI 1-Step Kit, featuring a non-WarmStart reverse transcriptase, exhibited significant non-template amplification.



Robust qPCR and RT-qPCR Quantitation and Detection

To further characterize Luna reagent performance and define sensitivity of absolute detection in this platform, we examined RNA and DNA targets of known copy number, evaluating quantitation and detection over a defined input range (10^8 down to 10 copies). For Luna RT-qPCR reagents (RNA input), we quantitated an RNA target from External RNA Controls Consortium (ERCC) RNA Spike-In Mix 1, which consists of 92 synthetic polyadenylated transcripts each at a defined concentration. For Luna qPCR reagents (DNA input), we quantitated a plasmid-encoded adeno-associated virus (AAV2) gene target. In both cases, quantitation was highly linear over the examined 8-log input range (AAV2 efficiency = 102%, R^2 = 0.998; ERCC efficiency = 100.7%, R^2 = 0.997). Even at a theoretical 10 copies per reaction, all replicates were detected, and replicate C_qs were highly reproducible (AAV2 CV at 10 copies = 1.6%, ERCC CV = 1.4%). This indicates that Luna reagents support detection and accurate quantitation even for targets that are present at very low copy number. The high sensitivity observed here is consistent with previous studies of Luna reagent performance in larger-volume reactions, in which low limits of quantitation (LoQs) and limits of detection (LoDs) were also observed, including a high success rate for single-copy detection (internal data).

To optimize low-copy detection, it should be noted that for nucleic acid samples at very low concentrations (e.g., ≤ 0.2 ng/µl), binding to plastic surfaces can sometimes deplete nucleic acids from solution (REF), leading to inaccurate quantitation or loss of detection. In such cases, addition of a carrier/blocking agent (e.g., non-template nucleic acid) or detergent (e.g., Tween 20) can mitigate this effect and yield improved results (Figure 4B; see legend). In addition, low-bind tubes and carboxy-coated Echo source plates can help prevent nucleic acid binding.

Dual WarmStart/Hot Start RT-qPCR Control for Room-temperature Stability

Most liquid handlers and automation platforms operate at or near room temperature, necessitating use of reagents that allow room temperature reaction setup. In addition, reagents that tolerate extended room temperature hold steps can enable greater flexibility and hands-off time in automated workflows. However, while RT-qPCR reagents commonly use hot start mechanisms to regulate *Taq* activity at room temperature, most do not similarly control reverse transcriptase (RT) activity, in part because typical RTs cannot tolerate the high-temperature denaturing activation step required for typical antibody-mediated enzyme control. In contrast, aptamer-based inhibition allows greater flexibility for defining activation temperature (NEB app note REF). Taking advantage of this, Luna OneStep kits employ unique dual enzyme control, via aptamer-based regulation of both *Taq* DNA Polymerase and Luna RT activities to prevent spurious amplification at room temperature. To examine the benefit of this dual enzyme control, one-step RT-qPCR reactions were assembled and run either immediately or after a 24-hour incubation at room temperature (Figure 3). No non-specific amplification was detected in reactions containing NEB Hot Start *Taq* and Luna WarmStart RT (Figure 3A). In contrast, clear evidence of non-specific amplification was detected after 24 hours when using a more typical RT-qPCR reagent that employs only single-enzyme control via a Hot Start *Taq* (Figure 3B). The added level of control imparted by Luna WarmStart RT can thus improve tolerance to both setup and extended incubations at room temperature, a critical feature for optimal performance in automated workflows.

A Complete Workflow for Automated, High-Throughput Direct-from-cells One-Step RT-qPCR

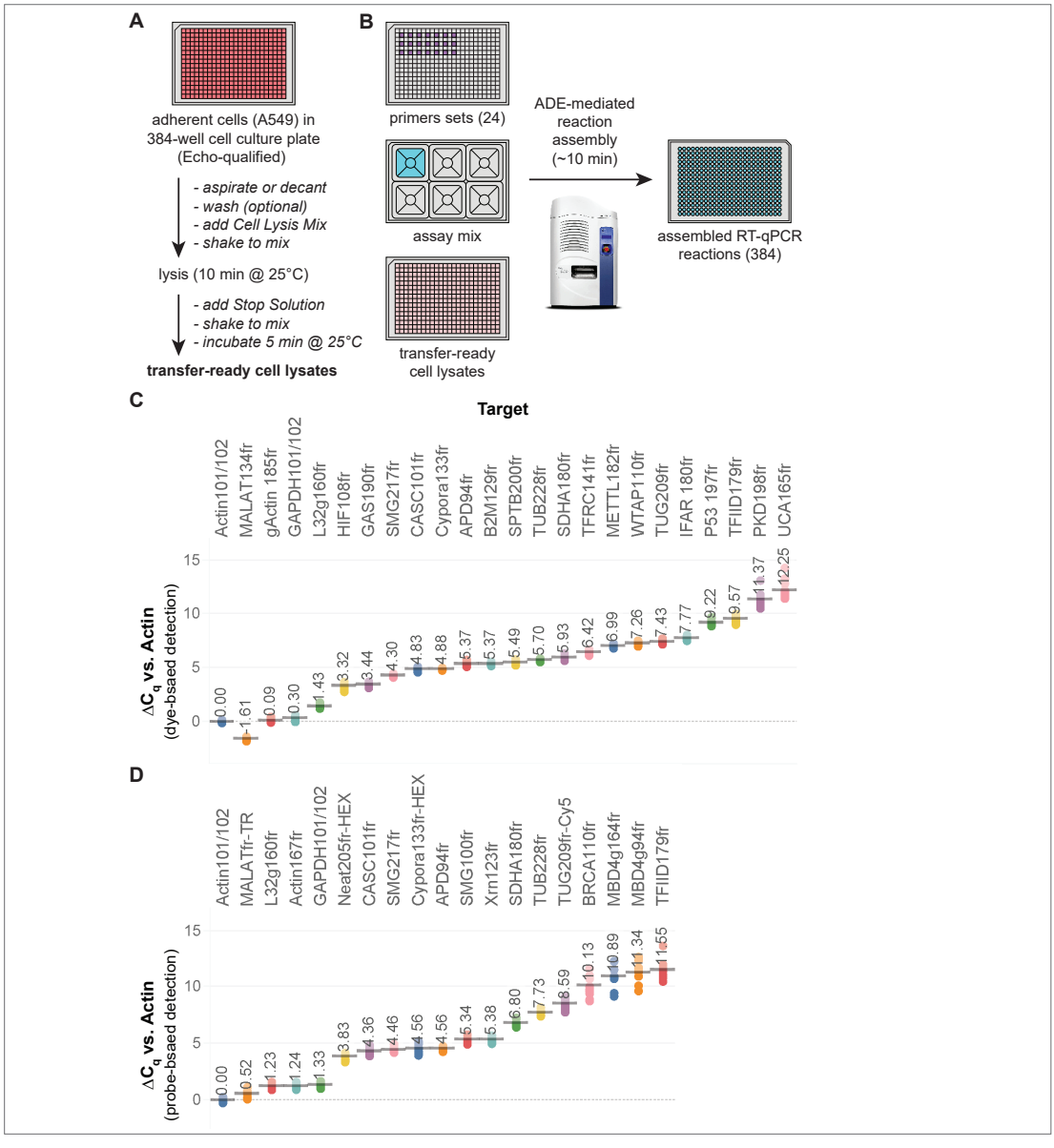
Direct analysis of cell culture lysates by RT-qPCR can offer a fast, convenient alternative to traditional one-step RT-qPCR by avoiding the need for a separate RNA purification step. The NEB Luna Cell Ready One-Step Kits and Lysis Module offer robust solutions for direct-from-cells RT-qPCR applications. To enable convenient automation of these workflows, we adapted the Luna Cell Ready Lysis Module protocol for direct use in cell culture-compatible Echo-

qualified source plates (Figure 4A). This required only minor changes to the standard Cell Ready 96/384-well protocol: lysis was carried out at room temperature (not 37°C), and plates were kept at room temperature after lysis (not placed on ice) to allow Echo-mediated lysate transfer (see Appendix for protocol details). The produced lysates were fully compatible with Echo-mediated acoustic liquid transfer (Table 1).

Using our adapted lysis protocol, we next tested performance by examining expression of 24 genes in A549 (human lung carcinoma) cells. Luna Cell Ready One-Step RT-qPCR reactions were assembled rapidly via automated ADE, with direct transfer of sample lysates from Echo-qualified culture plates into the reactions (Figure 4B). 12 replicates reactions were carried out for each target (using 6 replicate samples and 2 technical replicates per sample), with additional No-RT and non-template (NTC) controls. Results were highly reproducible between technical and biological replicates (Figure 4C). Even for the least abundant gene targets, the coefficient of variation (CV) for replicate C_q values for was ≤ 2.5%, and the average CV was < 1.3%. To further verify performance, we examined 19 genes additional genes using Echo-mediated assembly of Luna Cell Ready Probe One-Step RT-qPCR reactions (probe-based rather than dye-based detection). Results were again highly reproducible (Figure 4D), indicating robust performance of the automated Luna Cell Ready workflows.

FIGURE 4:
A complete workflow for direct one-step RT-qPCR from Echo-qualified cell culture source plates

A. A549 (human lung carcinoma) cells were seeded to 384-well Echo-qualified source plates at 1000 cells per well, grown overnight and lysed for 10 min at room temperature using the Luna Cell Ready Lysis Module (NEB #E3032). **B.** Lysate was then transferred directly into Echo-assembled Luna One-Step Universal RT-qPCR reactions. **C.** Relative expression of 24 gene targets was examined, with 6 biological replicates (cell culture source plate wells) and 2 technical replicates per target (288 reactions total). Dots indicate replicates (12 per target); lines and associated labels indicate average C_q (top) or ΔC_q vs. Actin (bottom) for each target. **D.** In a similar experiment, relative expression of an additional 19 gene targets was examined via probe-based quantitation using the Luna Cell Ready Probe One-Step Kit (NEB #E3031).



Conclusion

Luna qPCR and One-Step RT-qPCR reagents offer strong performance using typical reaction parameters, and are also fully compatible with Echo-mediated acoustic liquid handling, yielding linear, accurate quantitation, sensitive detection, exceptional reproducibility, and robust room temperature stability during automated workflows. Echo-mediated reaction setup in turn enables fast, accurate, high-throughput assembly of qPCR experiments, including those at a scale or level of complexity that would be impractical with manual setup. Finally, an adapted Luna Cell Ready protocol allows direct lysis of cells in Echo-qualified cell culture source plates, enabling a convenient, automated, high-throughput workflow for direct one-step RT-qPCR analysis of cell lysates. Taken together, these features make Luna qPCR reagents a strong choice for automated high-throughput qPCR and RT-qPCR applications.

Content approval code: AAG-6553APP03.20

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