Improving qPCR Reliability: Automated RNA Normalization and Plate Setup on PIPETMAX®

Application Note TRANS915

Normalization of RNA concentrations before reverse transcription reduces error in qPCR experiments and is recommended by the MIQE guidelines. PIPETMAX Normalization Assistant delivers an easy-to-use software and hardware solution to automate this task without compromising RNA integrity. PIPETMAX qPCR Assistant generates standardized and reproducible methods to automate qPCR plate setup. This powerful yet easy-to-use system integrates into your workflow, providing reliable pipetting and verifiable results while eliminating bookkeeping errors and user variation.

Introduction

Quantitative real time PCR, often coupled with reverse transcription, is a powerful technique for detecting differences in the copy number of nucleic acids in biological samples. qPCR can be applied for analysis of gene expression, copy number variation, detection of viruses, food safety testing and more.

Each step in the extremely sensitive qPCR procedure can potentially contribute to experimental error. Accurate and precise pipetting can minimize sources of variability, and it is recommended to "reduce technical errors in PCR reaction setup by standardizing (robotize if possible)…" [1] To permit replication of experimental techniques between laboratories, guidelines[2] have been established for the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE).

MIQE recommends normalizing the amount of input RNA used for reverse transcription (RT). Variable amounts of RNA can lead to differences in the efficiency of the RT reaction and in the quantification cycles (Cq) values observed in the subsequent real time PCR step.[3]

Normalizing nucleic acid concentrations requires precise calculations and repetitive pipetting. The manual procedure can be tedious and error-prone. PIPETMAX Normalization Assistant (Figure 1) delivers reproducible automated pipetting, while reducing record-keeping errors and user-to-user variability.

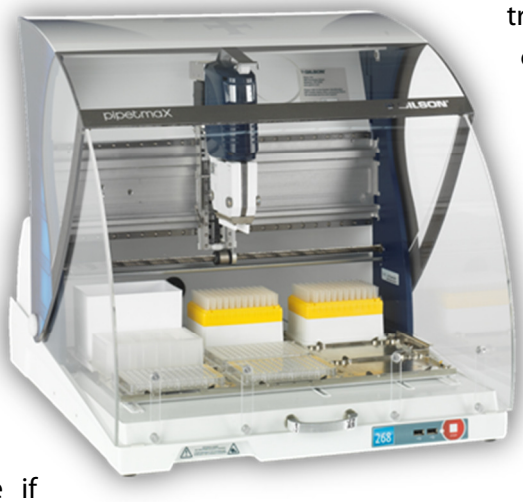

Figure 1. PIPETMAX *Normalization Assistant includes an automated pipetting workstation and easy to use software interface that runs on a tablet or PC. The system supports many types of labware such as microfuge tubes, reservoirs, strip tubes, 96 well plates and 384 well plates.*

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Materials & Methods

Cell Culture

Human liver carcinoma cells (HepG2) were a generous gift from Prof. Firouz Darroudi (Leiden University Medical Centre, Department of Toxicogenetics, Leiden, The Netherlands). Cells were grown under standard conditions at 37°C with 5% CO_2 in William's Medium E (Sig-(Sigma) supplemented with 15% fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin.

"PIPETMAX, a robotic liquid handling platform with PIPETMAN inside, automates routine pipetting and allows scientists to focus on science."

HepG2 cells were seeded on T-25 plates (Corning Costar Corporation) at a density of 10^6 cells per plate and incubated for 24 h at 37 °C and 5 % CO_2 in a humidified atmosphere. Cells were washed with 1x PBS, then exposed to one of two non-cytotoxic concentrations (0.01 or 1 µg/mL) of chemical compounds A-D and incubated for 24 h. An untreated control was included for each compound. After the treatment, cells were washed twice with 1x PBS and total RNA was isolated using TRIzol® reagent with minor modifications to the manufacturer's protocol as follows: glycogen (20 µg/mL) was added to the cell lysate, and the RNA was incubated with isopropyl alcohol overnight at -20°C to precipitate. All solutions for RNA isolation were prepared in RNase-free water.

Nucleic Acid Normalization

RNA was quantified using A260/A280 data collected using a Nanodrop™ spectrophotometer (Thermo). RNA concentrations were normalized with PIPETMAX® Normalization Assistant (Gilson, Inc.); the fixed sample volume on source plate scenario was used and buffer was used as diluent. After normalization, RNA was again quantified using a Nanodrop™ spectrophotometer. Agilent 2100 Bioanalyzer measurements were taken before and after normalization to assess RNA integrity.

Gene Expression Analysis

Gene expression was quantified by RT-qPCR. First, 2 µl of RNA that had been normalized in the previous step was transcribed to cDNA in a 25 µl total volume using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's protocol.

Serial dilutions of samples were performed on PIPETMAX. Master mixes were prepared in a separate clean room, as required by the SOP for the laboratory. The qPCR plate was prepared using PIPETMAX qPCR Assistant and the PIPETMAX 268 automated pipetting instrument (Gilson, Inc.).

Reagents included a FastStart Universal Probe Master mix (Rox) (Roche) and CDKN1A (cyclin-dependent kinase inhibitor 1A) Hs00355782_m1; and MYC (v-myc avian myelocytomatosis viral oncogene homolog, Hs00153408_m1) exon-spanning Taqman Gene Expression Assays. GAPDH was used as a reference gene. Reactions (in triplicate) were cycled in a HT7900 Real Time PCR System (Applied Biosystems, USA) according to manufacturer's protocols. Genomic DNA contamination was controlled by no-RT controls. The standard curve method was used for relative gene expression quantification. Transcript accumulation of each gene was normalized to GAPDH gene expression.

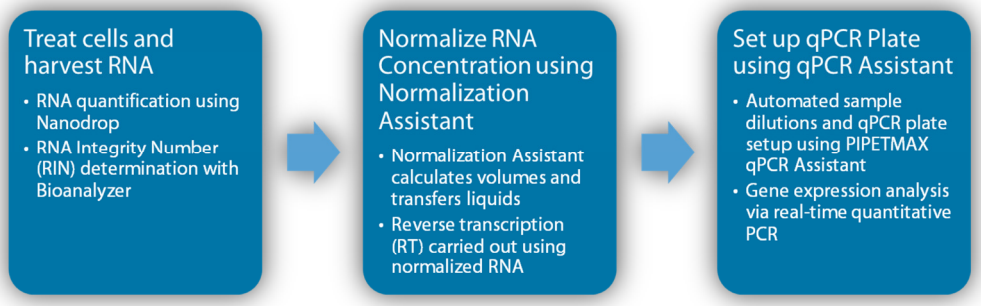

Figure 2. PIPETMAX Normalization Assistant and qPCR Assistant integrate into your qPCR workflow, providing time savings and enabling verifiable science.

Results and Discussion

PIPETMAX® is a robotic laboratory assistant with motorized PIPETMAN® technology. The software interface, TRILUTION® micro 2.0, includes wizard-style assistants that automate common laboratory procedures including normalization of nucleic acid concentrations and qPCR reaction setup. This application note presents data from samples processed using PIPETMAX Normalization Assistant and PIPETMAX qPCR Assistant.

Normalization of RNA Concentrations

In this study, human liver (HepG2) cultured cells were treated with a panel of four compounds (A-D), each at one of three doses (0, 0.01 or 1 µg/mL). The workflow of the experiment is shown in Figure 2.

After RNA isolation and quantification, PIPETMAX Normalization Assistant was used to normalize RNA concentrations. Values for initial concentrations were imported into the software from a CSV file, using the wizard-style interface (Figure 3). Whereas manual normalization of nucleic acid concentrations requires manual adjustment of the pipette at each step, a tedious and error-prone process, Normalization Assistant software calculates what volumes are required to accomplish the necessary dilutions and moves the required liquid to the correct location.

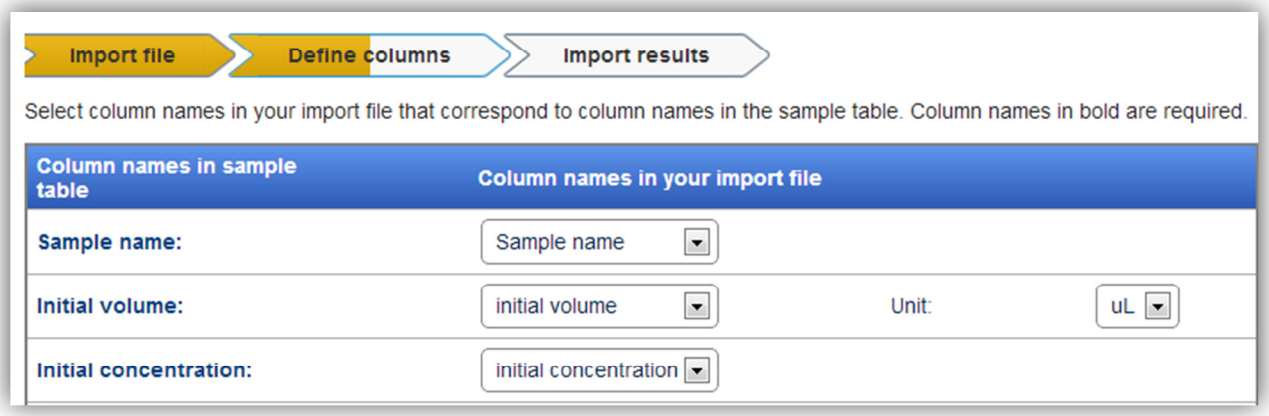

Figure 3. PIPETMAX Normalization Assistant supports import of sample lists as CSV (comma separated values) files. Sample name, initial volume, initial concentration, and position can be imported, as well as optional fields for tracking nucleic acid quality (such as 260/280 ratio), comments, and plate number for larger experiments.

PIPETMAX Normalization Assistant allows the user to define and apply filters in order to skip samples that don't meet minimum concentration requirements, or whose A260/280 ratios fall outside of the user-defined acceptable range. Documentation files detail the sample import, initial and final concentration and volume, any intermediate dilutions that were performed, and which (if any) samples were skipped due to quality or concentration. These documents can be incorporated into laboratory information systems (or printed for lab notebooks), thereby avoiding manual bookkeeping errors and improving reliability.

The results in Figure 4 show the RNA concentration before and after normalization with PIPETMAX Normalization Assistant. After normalization, all RNA samples were at the same approximate concentration. This enabled the use of a fixed volume of normalized RNA in the subsequent reverse transcription step, as recommended in the MIQE guidelines. RNA was analyzed with an Agilent Bioanalyzer before and after normalization (data not shown). RIN (RNA integrity numbers) remained >9.5, indicating that RNA integrity was maintained throughout the normalization process.

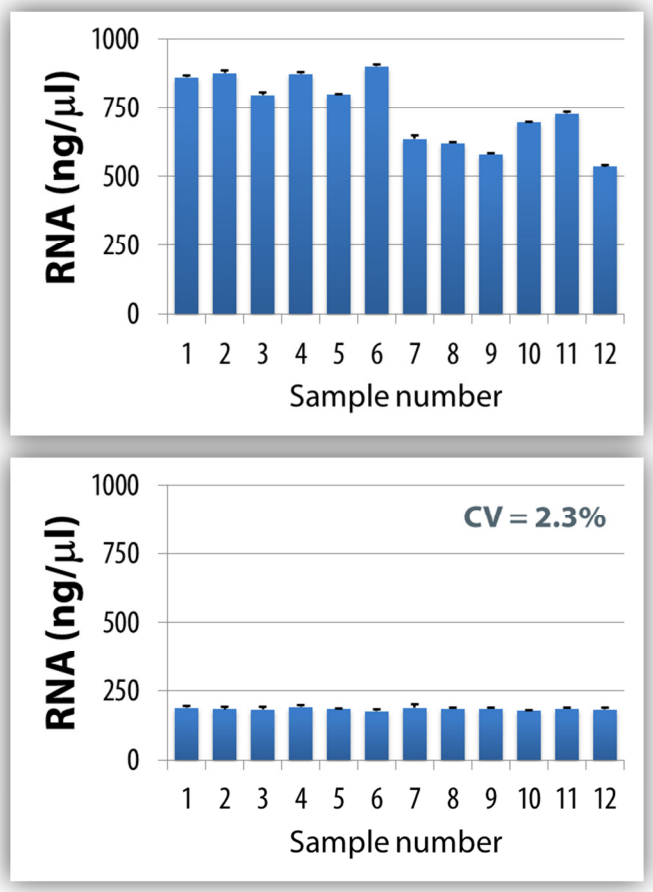

Figure 4. Human RNA samples were normalized using PIPETMAX Normalization Assistant. Graphs show the concentration of each sample before (top) and after (bottom) normalization. Error bars show range for Nanodrop concentration measurements.

qPCR for Gene Expression Analysis

In order to determine the effect of the treatment on gene expression, quantitative real-time PCR was carried out. PIPETMAX qPCR Assistant was used to perform serial dilutions of the samples, and to pipette all necessary reagents into the 384 well plate for cycling.

The wizard-style interface allows users to establish experimental parameters including number of replicates, dilution factors, and primer/template combinations. qPCR Assistant generates run documentation that can be incorporated into laboratory notebooks, including master mix recipes, a detailed pipetting guide, list of labware used in the experiment, and graphical representations of reaction plate configuration. Selections from the pipetting guide are shown in Figure 5.

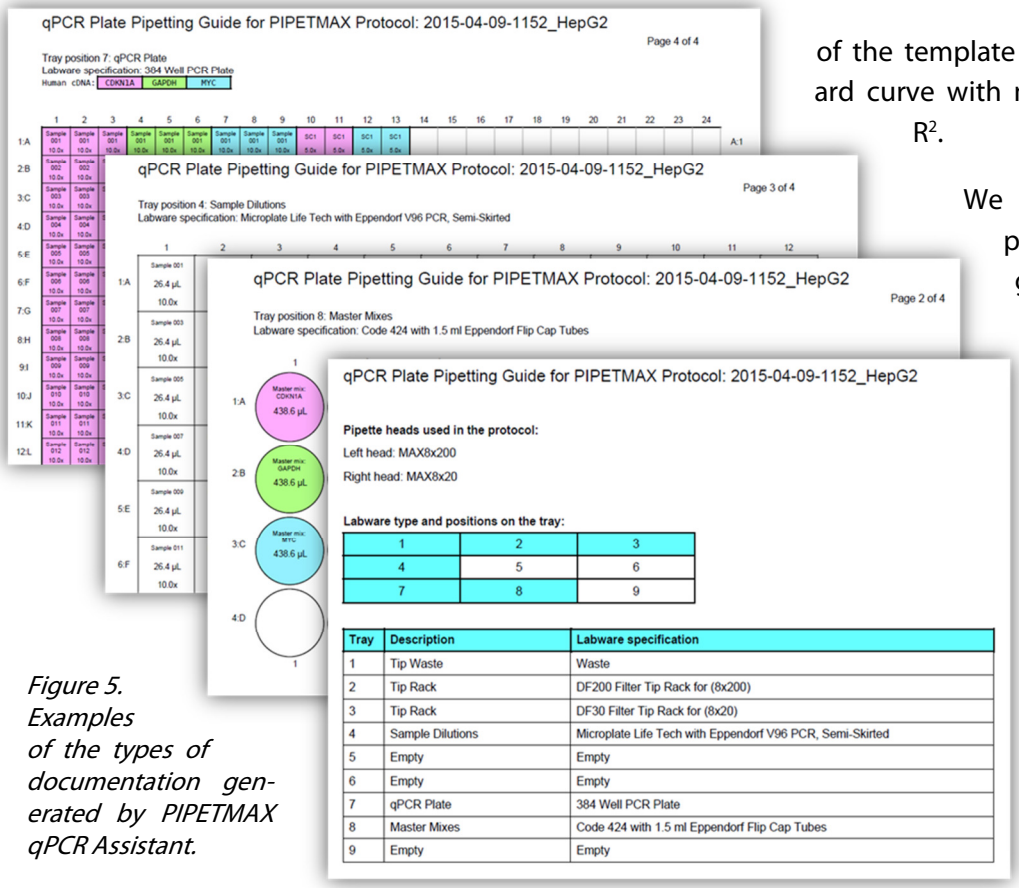

Figure 5. Examples of the types of documentation generated by PIPETMAX qPCR Assistant.

qPCR Assistant helps to reduce error in experimental setup, by automating the process of pipetting into 96 and 384 well plates. Additionally, the software generates thermocycler run files that are compatible with all major real-time PCR instruments; this permits direct import of the sample information into the thermocycler and eliminates the need for data entry at that step, resulting in significant time-savings for the user while assuring traceability of the data.

In this study, PIPETMAX® qPCR Assistant was used to generate sample dilutions for standard curves. As shown in Figure 6, ten-fold dilutions of the template resulted in a standard curve with near-ideal slope and R^2.

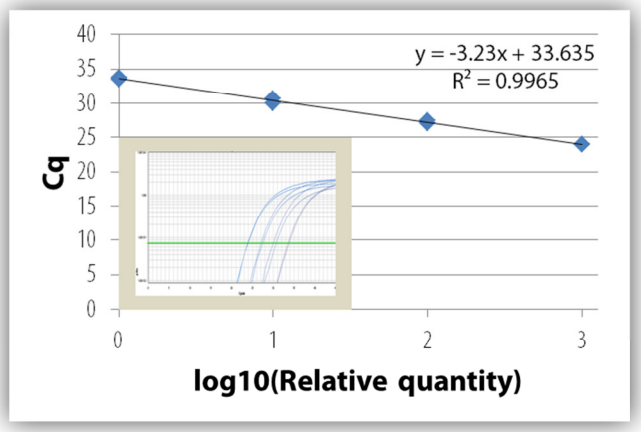

Figure 6. Standard curve for CDKN1A amplicon. Ten-fold dilutions of template and reaction setup were carried out using PIPETMAX qPCR Assistant. The slope is close to the ideal of -3..32

We examined the expression of two genes, MYC and p21, also known as CDKN1A. Gene expression data are expressed relative to a reference gene (GAPDH) and relative to the untreated control.

Figure 7 shows the results from the gene expression analysis. Cells were exposed to one of four compounds, at one of three doses as described. Compound A did not induce a significant change in gene expression. Compounds B, C, and D showed a dose response, and increased expression of CDKN1A at the highest dose was correlated with decreased expression of MYC, consistent with previously reported results.[4,5]

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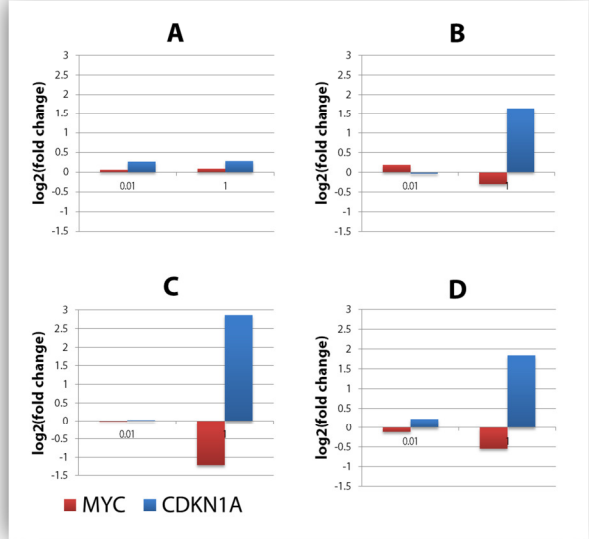

Figure 7. Results from RT-qPCR experiment. Relative transcript abundance was determined for myc and CDKN1A from cells treated with 0.01 or 1 µg/ml of compounds A – D. The Y axis shows log2 (fold change) in gene expression relative to the reference gene (GAPDH) and relative to untreated cells.

PIPETMAX Normalization Assistant and PIPETMAX qPCR Assistant fit into the qPCR workflow and provide standardized and reliable methods to help your lab meet the MIQE publication requirements. These easy to use automation solutions reduce user variability and improve the verifiability of your experiments. Output files document the labware used, sample names, positions, initial & final concentrations and whether any samples were skipped due to user-defined filters. This documentation can be easily incorporated into laboratory record keeping procedures.

References

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Acknowledgements

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Summary

- PIPETMAX Normalization Assistant can normalize RNA concentrations before RT-qPCR, as recommended by MIQE guidelines, while maintaining RNA integrity throughout the procedure.
- PIPETMAX qPCR Assistant automates plate preparation for qPCR, including preparation of master mixes, standard curve and sample dilutions, and reaction setup. The easy-to-use wizard-style interface guides the user through experimental parameters including selection of controls, dilution factors, and replicates.
- PIPETMAX, a robotic liquid handling platform with PIPETMAN inside, automates routine pipetting and allows scientists to focus on science.

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