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Biomek i7 Hybrid Automated Workstation Enables Automation of the Promega GoTaq Probe 2-Step RT-qPCR System

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Abstract

Reverse transcription quantitative PCR (RT-qPCR) is widely used for quantifying the amount of a specific RNA sequence in a sample. This method has proven extremely useful in a variety of workflows, including gene expression quantification and the diagnosis of infectious diseases, such as RNA viruses. Laboratory automation offers many advantages over manually preparing RT-qPCR assay plates, such as increased throughput and minimized likelihood of user-introduced error. Here we describe automation of the Promega GoTaq Probe 2-Step RT-qPCR workflow using a Biomek Hybrid Automated Workstation. The automated method performed equivalently to manually performed assays, as 1 ng RNA input produced C_T values of 21.60 and 21.68 for manual and automated methods, respectively. Additionally, the automated method maintained the wide dynamic range of the GoTaq kit. RNA input could be accurately quantitated over the entire range of inputs tested (10 ng to 3 pg) with an R^2 value of 0.9986. Together the data presented here shows that the Biomek i7 Hybrid Automated Workstation can automate the RT-qPCR workflow, providing high quality results, while reducing user hands-on time and the possibility of user-introduced error.

Introduction

The discovery of the polymerase chain reaction (PCR) in the early 1980s revolutionized biomedical research by enabling the routine amplification of a specific sequence of DNA to generate billions of copies.[1] PCR has had widespread impact in the fields of genomics, DNA sequencing, cloning, forensics, and infectious disease, to name a few, and has opened the door to numerous variations of the general PCR workflow. These variations include quantitative PCR (qPCR), reverse transcription PCR (RT-qPCR), Multiplex PCR, Inverse PCR, and digital PCR, each of which has unique utility within the scientific community. One method that has become a critical tool is quantitative PCR (qPCR).[2]

The qPCR method was first described in the early 1990s and allows real-time, relative quantitation of the number of copies of a specific DNA sequence within a given sample.[2] Quantitation is achieved by monitoring the fluorescence of reporter dyes following each cycle of a PCR protocol. In general, there are two types of qPCR setups available to users: probe-based (e.g. TaqMan) and dye-based (e.g. SYBR). Dye-based methods employ an intercalating chemical that nonspecifically binds to DNA and becomes fluorescent. As more DNA from the genomic region of interest is generated during each PCR cycle, an increase in fluorescence is observed.[2] Probe-based setups use a sequence-specific, short DNA probe that is complementary to a region within the genomic location of interest. This probe is labeled with a fluorophore and a quencher, such that when the probe is intact, no fluorescence is observed. As the region of interest is amplified during PCR cycles, the probe is displaced and degraded by the exonuclease activity of the polymerase.[3] This frees the fluorescent probe from the quencher, and an increase in fluorescence is measured (Figure 1A).

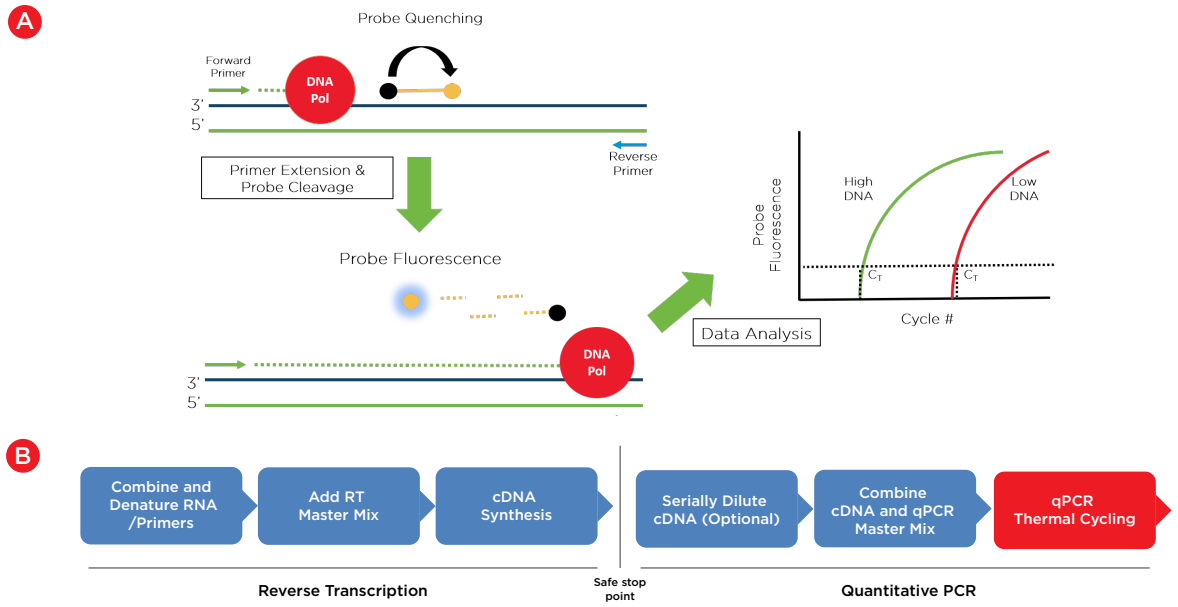

Figure 1. A) Mechanism of hydrolysis probe-based qPCR (TaqMan). B) Workflow of Promega GoTaq Probe 2-Step RT-qPCR System automated on the Biomek i7 Hybrid Automated Workstation. Steps highlighted in blue were automated and performed via Biomek, while the red qPCR step was performed off-line using a QuantStudio 6 Flex (Applied Biosystems).

In addition to being used for DNA quantitation, qPCR can be used to quantify the amount of a specific region of RNA within a sample using RT-qPCR. In this workflow, RNA is first converted to cDNA using a reverse transcriptase enzyme, followed by typical qPCR thermal cycling and fluorescence measurement.[4] RT-qPCR can be performed using various commercially available kits. These kits can be subdivided into one-step and two-step methods. In one-step RT-qPCR the reverse transcriptase and all required qPCR reagents are combined into a single master mix that is added to an RNA sample. The isothermal RT reaction is performed, the RT enzyme is then denatured at high temperature, and qPCR immediately follows. This all occurs in a single tube/well. This has the advantage of being faster and easier to set up but limits the number of target genes that can be assessed from a single RNA sample and limits flexibility in the qPCR portion of the assay. In a two-step method, the RT reaction is first performed to generate cDNA. This cDNA is then used as input for the subsequent qPCR which is performed in a separate tube or well. This allows the user to perform qPCR on a single cDNA sample at numerous genetic loci by varying the primers/probe. One commercially available kit is the Promega GoTaq Probe 2-Step RT-qPCR system.[5] The workflow for this kit is outlined in Figure 1B. As the Promega two-step qPCR method has various steps requiring accurate pipetting and precise timing, automation of the protocol could decrease the amount of user hands-on time, while increasing throughput. Here we sought to automate this method using a Biomek i7 Hybrid Automated Workstation (Figure 2).

The Biomek i7 Hybrid Automated Workstation is an automated liquid handler that is capable of efficiently performing the complex liquid handling steps of qPCR workflows. This minimizes the number of required user interactions and increases walkaway time, freeing the operator to attend to other laboratory tasks. The multichannel pod can be equipped with a 96-well head that can accurately pipette 1 to 1200 µL or a 384-well head that is accurate over the range of 0.5 to 60 µL. Additionally, the 8-channel Span-8 pod is accurate from 1 to 1000 µL. The Biomek i7 Hybrid Automated Workstation supports 45 deck positions and can be directly fitted with orbital shakers, heating/cooling Peltiers, and tip-washers for plate and sample processing. Further, depending on user needs, the Biomek i7 Hybrid Automated Workstation supports integration with other automated plate handling instruments, such as thermal cyclers, incubators, barcode readers, washers, multimode plate readers, centrifuges, and more. Here we show that automated processing of the GoTaq Probe 2-Step RT-qPCR System using a Biomek i7 Hybrid Automated Workstation provides excellent results that are equivalent to manually processed samples. The automated workflow can reduce the hands-on time and the possibility of user sample handling errors.

Figure 2. Biomek i7 Hybrid Automated Workstation

Methods

Reverse Transcription

Initial experiments focused on the automation of cDNA generation via the RT reaction. This was accomplished in a 96-well PCR plate using an automated method with two heating/cooling peltiers, each of which had 96-well block adaptors. This allowed the instrument to incubate the RT reaction plate for the appropriate time at the correct temperature at each step. The deck layout for the RT reaction performed using the Biomek i7 Hybrid Automated workstation is presented in Figure 3A. On the deck of the instrument, in one well of a 96 well PCR plate (BioRad), 15.5 µL of nuclease-free water was combined with 14.5 µL of 50 ng/µL human total RNA (Applied Biosystems), and 5 µL each of the Oligo dT and Random primer stocks provided in the GoTaq kit, for a total volume of 42 µL. The plate was then sealed and incubated at 70°C for 5 minutes using an on-deck integrated static peltier. The plate was then transferred to a second static peltier and incubated at 4°C for 5 minutes, followed by a 30 sec centrifugation step at 1500 rpm in an integrated microplate centrifuge (Agilent). During plate incubations, the RT Master Mix was assembled according to manufacturer instructions; 49 µL of nuclease-free water was combined with 40 µL reaction buffer, 16 µL $MgCl_2$, 10 µL nucleotide mix, and 5 µL RNasin.[5] To 83 µL of this mix, 7 µL of Reverse Transcriptase enzyme mix was added, and to the remaining 37 µL of mixture, water was added in lieu of enzyme to serve as negative control. After master mix preparation, 7 µL of denatured RNA/primer mix was moved into an adjacent well of the PCR plate and 13 µL of negative control master mix was added for a total volume of 20 µL. To the well now containing 35 µL of RNA/Primer mix, 65 µL of RT Master Mix was added for a total volume of 100 µL. The plate was then re-sealed and spun at 1500 rpm for 30 sec in the on-deck centrifuge. Finally, the plate was incubated on the instrument deck at ambient temperature (23°C) for 5 min (annealing), moved to a static peltier set at 42°C for 45 min (extension), and then moved to a second static peltier set at 70°C for 15 min (enzyme denature) (Figure 3A). The plate was stored at -20°C until the day qPCR was performed.[5]

Manual vs Biomek qPCR

In order to perform the qPCR reaction assembly, the deck of the Biomek i7 Hybrid Automated workstation was set up as shown in Figure 3B. The Master Mix for qPCR was assembled according to manufacturer instructions by combining the following reagents: 600 µL 2X GoTaq Probe Master Mix (Promega), 60 µL 20X human GAPDH TaqMan Assay (Applied Biosystems), and 240 µL nuclease-free water.5 The cDNA generated above was diluted 30-fold in nuclease-free water, and water alone served as no template negative control. 15 µL Master Mix was added to each well using the Span-8 (Biomek) or by hand (Manual), the plate was spun at 1500 rpm for 30 sec using the on-deck microplate centrifuge, and 5 µL of cDNA or negative control was added to the plate either by hand or using the Span-8. The plate was spun at 1500 rpm for 30 sec, sealed, and transferred to a QuantStudio 6 Flex (Applied Biosystems). Thermal cycling was performed according to manufacturer guidelines: 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec (Denature)/ 60°C for 1 min (Anneal/Extend).[5]

cDNA Titration qPCR

Master Mix was assembled as described above and cDNA from RT reaction was subjected to an eight point, half-log serial dilution in nuclease-free water from 10 ng to 3 pg per well using the Span-8 pod on the Biomek i7 Hybrid Automated Workstation. 15 µL of qPCR Master Mix was added to the plate using the Span-8 pod, followed by the addition of 5 µL cDNA using the Biomek Multichannel pod. The plate was spun at 1500 rpm for 30 sec, sealed, transferred to a QuantStudio 6 Flex (Applied Biosystems), and thermal cycling was performed as described above.

Data Analysis

Cycle threshold (C_T) and reporter-normalized fluorescence (ΔRn) values were calculated by QuantStudio Real-Time PCR Software Version 1.3 (Applied Biosystems). Biomek versus manual data (Figure 4) are representative of eight technical replicates and presented as mean ± standard deviation. The RNA input presented in Figure 5 was caulcated based on the dilution factor of the initial RNA used in the RT reaction. These data are representative of the mean ± standard deviation of three technical replicates. Graphs and line fitting were generated using Prism 9 software (GraphPad). In some cases, error bars are too narrow to be visible.

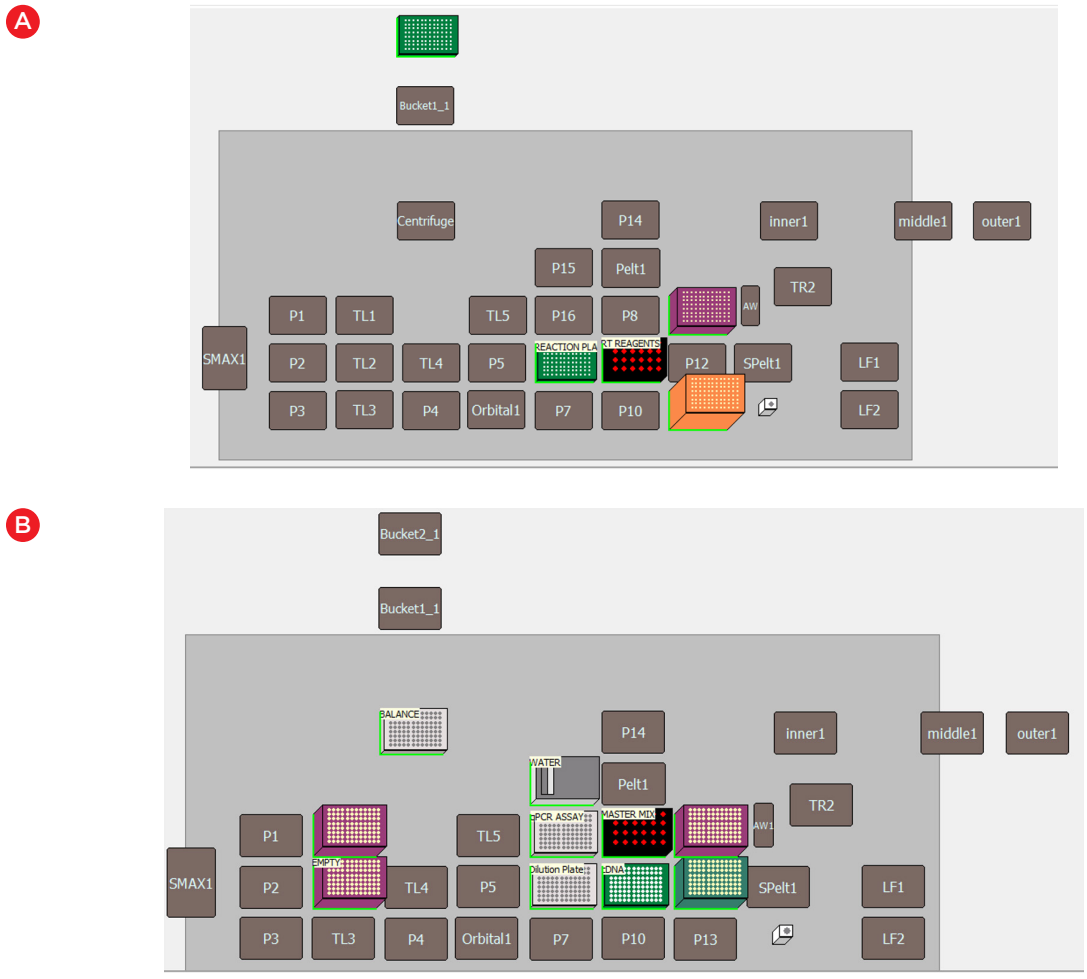

Figure 3. Deck layout for Biomek i7 Hybrid Automated Workstation Promega GoTaq Probe 2-Step RT-qPCR method: RT reaction (A) and qPCR (B). The combined automated method used the following components: 2 tip-loading ALPs, 7 1X1 ALPs, 1 trash bin, 2 heating/cooling peltiers, and an Agilent Microplate Centrifuge.

Results and Discussion

Commercially available total human control RNA was selected to serve as the template for the RT reaction. This was combined with the RT reaction primers using the Span-8 pod, denatured, and RT master mix was added using the Span-8 pod. RNA/primers were annealed, extended, and then the enzyme was inactivated according to manufacturer instructions.

Following automation of the RT reaction, focus was shifted to automation of the qPCR section of the workflow. In order to validate the automated method, results from manually assembled qPCR wells were compared to wells generated using the Biomek i7 Hybrid Automated Workstation. GAPDH was selected as the gene of interest, as it is often employed as a reference gene and has a TaqMan primers/probe pair commercially available. 1 ng/well of cDNA generated via the automated RT reaction was used as the template and was combined with qPCR Master Mix either by hand or using the Span-8 pod. The results following qPCR thermal cycling are presented in Figure 4. For the GAPDH assay, FAM fluorescence from the TaqMan probe was normalized to the passive reference fluorophore (CXR) provided in the GoTaq kit. In order to quantitate gene expression, the QuantStudio Real-Time PCR Software calculated a cycle threshold (C_T) for each well. The automated method was accurate as compared with manually generated results. In both manually and Biomek prepared wells, a robust response is observed, and the raw traces are nearly identical (Figure 4A). The calculated C_T values were also in excellent agreement with C_T ± SD values of 21.60 ± 0.06 and 21.68 ± 0.08 for manual and Biomek, respectively. As the deviation values suggest, the precision for the automated method was excellent and on par with the manual assay (Figure 4B). Together this data showed that the GoTaq Probe 2-Step qPCR system was highly amenable to automation using a Biomek i7 Hybrid Automated Workstation. The automated method provided excellent results that were equivalent to assays performed by hand.

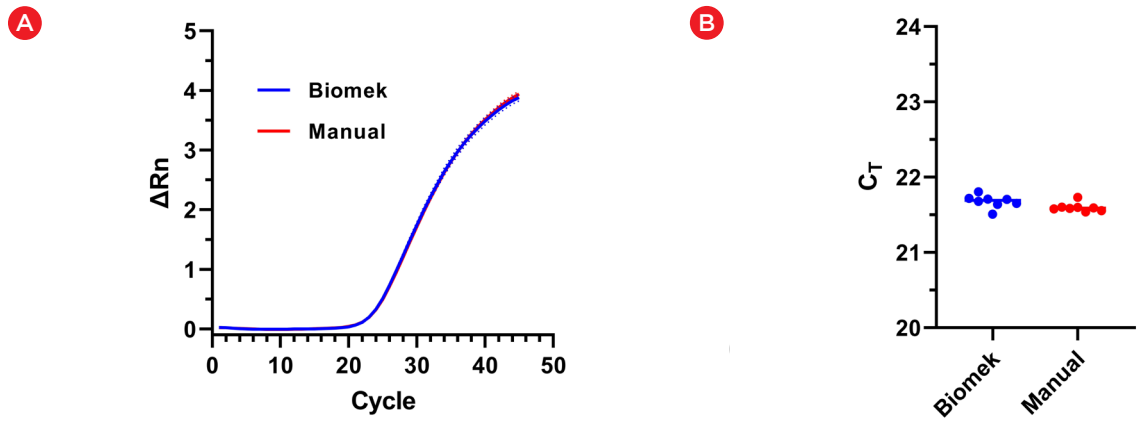

Figure 4. Comparison of manual versus Biomek i7 Hybrid Automated Workstation quantification of GAPDH from total human RNA using GoTaq Probe 2-Step RT-qPCR. (A) Raw traces of cycle number vs normalized TaqMan probe fluorescence. (B) C_T for each individual replicate used in the manual versus Biomek experiment. Data represents mean ± SD for eight technical replicates.

We next sought to ensure that the Biomek-enabled automated method could be used to quantify a wide range of RNA/cDNA concentrations, as the wide dynamic range is one of the advantages of the GoTaq qPCR system. To assess this, the cDNA generated in the RT reaction was serially diluted using the Span-8 pod in eight point, half-log (3.16-fold) dilutions (10 ng to 3 pg) followed by addition of qPCR master mix. The resulting data is shown in Figure 5. As expected, a concentration dependent shift in the raw fluorescence trace was observed (Figure 5A). The highest amount of RNA input (10 ng) produced a C_T value of 18.2 ± 0.1, while the lowest concentration tested (3 pg) had a C_T value of 30.33 ± 0.09. 1 ng RNA input gave a C_T value of 21.71 ± 0.07, which was very similar to the values observed in Figure 4. In order to analyze the dynamic range of the kit, the calculated C_T value was plotted as a function of RNA input quantity and analyzed using linear regression with GraphPad Prism (Figure 5). Excellent linearity (R^2 = 0.9986) was observed for this fit, indicating that the kit is suitable for RNA quantitation over the entire range of values tested, which was more than three orders of magnitude. Together this data showed that the automated method faithfully retained a key feature of the GoTaq kit, the wide dynamic range.

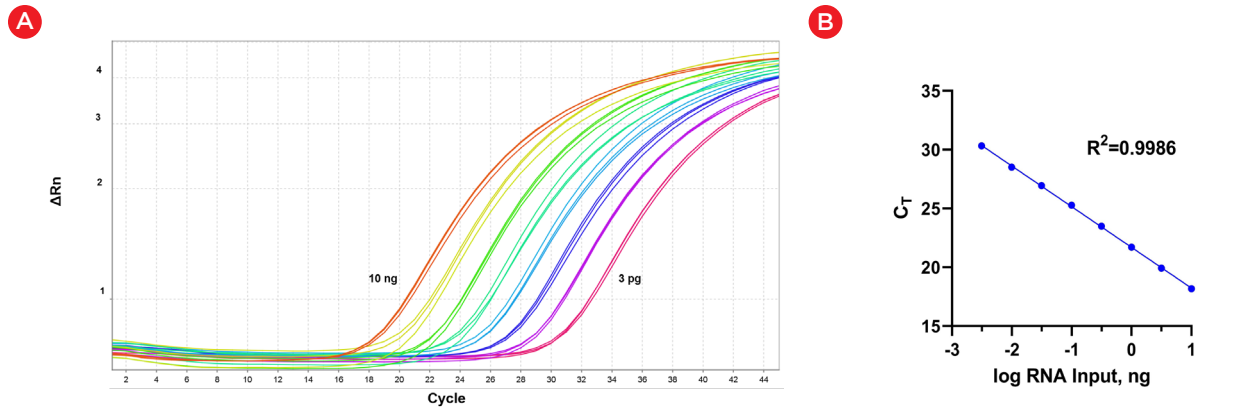

Figure 5. GAPDH RNA quantification following 8-point, half-log serial dilution from 10 ng to 3 pg per well. (A) Raw fluorescent trace of cycle number vs normalized probe fluorescence for each dilution. (B) C_T for each sample as a function of RNA input to assess dynamic range of GoTaq method automated via Biomek. Data represents three technical replicates of each concentration. SD error bars in B are too narrow to observe.

In addition to processing a single RT-qPCR plate like the method developed here, the Biomek i7 Hybrid Automated Workstation can support assembly of multiple plates for users requiring higher throughput. As can be seen in Figure 3, many of the 45 available deck positions are unoccupied in the automated RT and qPCR methods shown here. These positions could be used to hold additional assay plates. Further, users concerned with increasing throughput may be able to miniaturize the setup from 20 µL assay volume in 96-well format to 10 µL assay volume in 384-well format, as the kit manufacturer has previously demonstrated.[5] Though these method modifications are not explored in this report, alteration of the general method developed here should be straightforward and could drastically increase throughput while reducing the hands-on time required by the user.

Summary

RT-qPCR has become a widely employed method in the scientific community with a wide range of applications, from simple gene expression analysis to the detection of RNA-based infectious diseases. Due to the sensitive nature of the reagents and the high-throughput demanded by users, RT-qPCR is a prime candidate for laboratory automation. Here we sought to develop an automated method for processing of the Promega GoTaq Probe 2-Step RT-qPCR system. Our data showed that the automated method performed similarly to manual methods, while maintaining the wide dynamic range that qPCR users demand. At 1 ng RNA input, manual methods produced C_T values of 21.60 ± 0.06 and the automated protocol provided 21.68 ± 0.08. The automated method allowed accurate RNA quantitation from 10 ng down to 3 pg RNA input with an R^2 value of 0.9986. Together the data presented here shows that the Biomek i7 Hybrid Automated Workstation can automate the RT-qPCR workflow, providing high quality results with the opportunity for increased throughput, while reducing user hands-on time and the possibility of user-introduced error.

References

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Materials

Equipment	Manufacturer
Biomek i7 Hybrid Automated Workstation	Beckman Coulter Life Sciences
Static Heating/Cooling Peltier ALP	Beckman Coulter Life Sciences
Microplate Centrifuge	Agilent
QuantStudio 6 Flex	Applied Biosystems

Table 1. Instruments used

Reagents	Manufacturer	Part Number
TaqMan GAPDH Assay	Applied Biosystems	Hs02758991_g1
GoTaq Probe 2 Step RT-qPCR System	Promega	A6110
Human Total RNA Control	Applied Biosystems	4307281

Table 2. Reagents used

Consumables	#	Manufacturer	Part Number
MicroAmp Optical 96W-Reaction Plate	1	Applied Biosystems	N8010560
MicroAmp Optical Adhesive Film	1		4311971
Hard-Shell PCR Plate, 96 well, thin-wall	2	BioRad	HSP9641
Biomek i-Series, 1025 µL pipette, sterile, filtered	1	Beckman Coulter Life Sciences	B85955
Biomek i-Series, 50 µL pipette, sterile, filtered	2		B85888

Table 3. Consumables used per run

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