



Using the ELx405™ to Automate Washing of the QuantiGene® Reagent System

Abstract

Genospectra's QuantiGene® Reagent System is a technology capable of quantitatively measuring RNA without amplifying the target. This technology, based on branched DNA (bDNA), requires multiple hybridization steps followed by washing to remove unhybridized reagent. Here we describe the use of Bio-Tek's ELx405™ Microplate Washer to automate the wash steps of this unique technology.

Introduction

Quantitating and characterizing gene expression through RNA measurement is a commonly performed task in today's biomedical research. Typically RNA expression is examined through quantitative PCR, which requires extensive RNA purification and amplification. These methods are time consuming, expensive, and prone to errors. To rectify these problems, alternative methods to quantify RNA have been developed. One such method is the QuantiGene® Reagent System, which measures RNA directly from tissue homogenates, cell lysates or purified RNA without target amplification. The QuantiGene® Reagent System uses a series of hybridization steps in conjunction with branched DNA (bDNA) technology to amplify the measurement signal. Each hybridization step requires a thorough manual wash step to remove the unhybridized material. In order to increase throughput and repeatability of the assay, there was a desire to automate the wash steps employed by the assay. Here we describe the important wash parameters of the assay and demonstrate the ability of the ELx405 Microplate washer to automate the wash steps employed by the QuantiGene® Reagent System.



Figure 1. ELx405™ Select Microplate Washer.

The QuantiGene® Reagent System measures mRNA levels directly from crude lysates, tissue homogenates, or purified RNA. It utilizes branched DNA (bDNA) technology, which relies on cooperative hybridization between the target mRNA and a specific probe set. The probe sets consist of three types of oligonucleotides, Capture Extenders (CEs), Label Extenders (LEs), and Blocking Probes (BLs), whose sequences are selected based on the sequence of the target mRNA. The Capture Extenders (CEs) are approximately 40 nucleotides in length with roughly half of the sequence being complimentary to sections of the target mRNA and the other half complimentary to the Capture oligos immobilized onto the bottom of the Capture microplates. The Label Extenders (LEs) are also approximately 40 nucleotides in length, with half of the

sequence being complimentary to the target mRNA and the other half complimentary to a portion of the branched DNA amplifiers. The Blocking Probes (BLs) are complimentary to regions of the target mRNA not recognized by either the CE or the LE oligonucleotides.

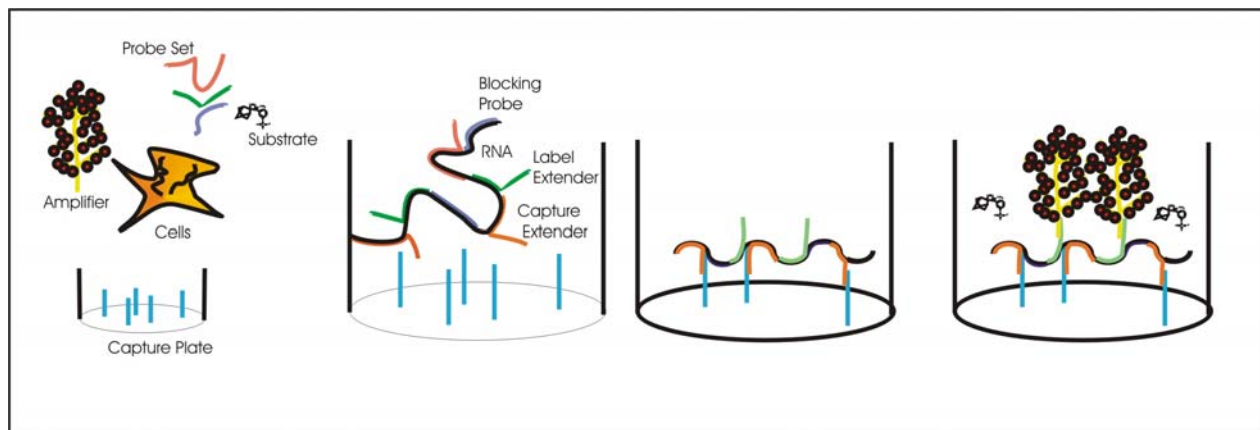


Figure 2. Overview of QuantiGene® Reagent System Technology.

The CEs provide the assay specificity by binding the target mRNA to the capture plate. Cooperative hybridization begins with the initial CE binding event, which tethers the mRNA to the capture plate. This makes it much more likely that the second and third CE oligonucleotides along the target mRNA will also bind the plate. The result is a very stable hybridization event where the target mRNA is attached at multiple locations to the capture plate. Signal amplification is the result of LEs interacting with the target mRNA and the branched Amplifier sequences. Each target mRNA is hybridized with multiple LEs oligonucleotides along its length, while each LE hybridizes with the trunk region of the branched amplifier sequence. The “branches” of each amplifier have binding sites for the label probe (alkaline phosphatase), which interacts with its substrate to provide a luminescent signal. Using this technology the amplification occurs with the signal rather than the target. The signal is proportional to the target mRNA and can be achieved without purification or amplification.

The ELx405™ Select is a multifunctional microplate washer that can wash both 96- and 384-well microplates without any mechanical changes required. This industry standard plate washer uses a patented manifold design which provides for independent control of aspirate and dispense tube location and height enabling bubble free fluid-dispense and overflow protection in 96- and 384-well plates. The dispense and aspiration manifolds are placed into two physically different parts that are arranged on top of each other. The lower manifold (dispense) is constructed in such a manner as to allow the tube from the above manifold (aspiration) to pass through and enter the well of the microplate. In order for the dispense pipe to be able to dispense fluid into a small well while the aspirate pipe is removing fluid from the same well, as is the situation when overflow and bottom washing are performed, the dispense pipe is tilted from vertical. This allows for the dispense tube to be offset from the center of the well, providing room for the aspiration pipe, yet still allowing the fluid jet to enter the well from the side. This canted design also has the added benefit of providing a swirling motion of the fluid resulting in a more vigorous wash.

Materials and Methods

Cell lysates from U937 cells in volumes ranging from 0 to 20 µl were transferred to separate Capture Plate wells in replicates of eight. These lysates had been previously calibrated to be equivalent to 100 cells per 1-microliter. To each well 10 µl of GAPD pooled probe set was added. Lysis working reagent was then added to bring the total volume of each well to 100 µl. The diluted GAPD mRNA specific probe set was prepared immediately prior to use according to

the kit instructions. Briefly, 5X-prepackaged probe sets (100 nM LE, 500 nM BL, and 250 nM CE) were diluted to 1X using TE (10 mM Tris 1 mM EDTA pH 7.5-8.0) buffer. A pooled probe set was then prepared by adding 200 µl of each probe set to 3400 µl of undiluted lysis mixture. The Capture Plates were sealed and incubated at 53°C for 16-20 hours (overnight).

The following day wells were washed either manually or with an ELx405™ Select Microplate Washer (Bio-Tek Instruments) as described in the washing instructions below. Wash buffer consisted of 0.1X saline-sodium citrate buffer (SSC) and 0.3% lithium lauryl sulfate in distilled H₂O. After washing, 100 µl of Amplifier working solution was added to each well and the plates were resealed and incubated for 60 minutes at 46°C. Amplified working solution was prepared immediately prior to use by diluting amplifier stock solution with amplifier/label probe diluent (both supplied in the QuantiGene kit) in a ratio of 1:1000 (stock:diluent). After incubation, the plates were again washed either manually or with an ELx405 Select and 100 µl of diluted label probe solution is added. The diluted label probe solution was prepared immediately prior to use by the dilution of label probe concentrate with amplifier/label probe diluent in a ratio of 1 µl concentrate to 1 ml of diluent (both supplied in the QuantiGene kit). The plates were again incubated for 60 minutes at 46°C. Following the second incubation plates were rewashed. After the final wash, working luminescent alkaline phosphatase dioxetane substrate was added, the plate was sealed and then incubated for 30 minutes at 46°C. Working substrate solution was prepared by adding 3 µl of a 10% lithium lauryl sulfate solution each 1 ml of the stock luminescent substrate. The luminescence signal was determined using an Lmax luminometer (Molecular Devices) set at 41°C.

ELx405 Washer Maintenance Procedures

Prior to using the washer for the first wash procedure outlined in the assay it is advisable to prime the wash buffer through the washer using the “New Buffer Prime” utility from the washer key pad. This will insure that fresh buffer has completely filled all of the fluid lines and any residual rinse water or other buffer has been evacuated. For all of the subsequent washes, the washer need not be primed. At the end of the day it is advisable that the washer be rinsed with deionized water and the washer set to run the “Over Night Loop” utility with water as the wash buffer. In addition it is advisable to follow the instructions in the Operators manual in regards to periodic performance testing of the washer.

Washing Procedures

When washing the plates manually, 290 µl of wash buffer was first dispensed to each well of the capture plate. Afterward, the entire contents of the well was removed by inverting the plate and forcefully dumping the fluid. The subsequent wash step received 390 µl of wash buffer and the contents again removed as described previously. Following the last aspiration the microplate was inverted and centrifuged for 60 seconds at 1000g to remove the residual fluids. Note that the wash step after the addition of label probe incubation, prior to the addition of substrate, required a total of three washes prior to centrifugation.

Program Name	D3	34	35
Link File			
File Type	Dispense	Wash	Wash
Method			
Number of Cycles		3	5
Soak/Shake		Yes	Yes
Soak Duration		10 sec	10 sec
Shake before soak		No	No
Prime		No	No
Prime Volume			
Prime Flow Rate			
Dispense			
Dispense Volume	290	390	390
Dispense Flow Rate	5	5	5
Dispense height	115	115	115
Horizontal dispense position	10	10	10
Bottom wash first	No	No	No
Bottom Dispense Volume			
Bottom flow rate			
Bottom Dispense Height			
Bottom Dispense Position			
Prime	No	No	No
Prime Volume			
Prime Flow Rate			
Aspiration			
Aspiration Height		32	32
Horizontal Aspiration position		-45	-45
Aspiration Rate		5	5
Aspiration Delay		0	0
Crosswise Aspirate		No	No
Crosswise Aspirate on			
Crosswise Height			
Crosswise horizontal position			
Final aspiration		Yes	Yes
Final aspiration delay		2000 msec	2000 msec

Table 1. ELx405 Select Washer settings for QuantiGene® Reagent System washing. A Dispense routine (D3) was linked to two different Wash routines (34 and 35) to create the Link files named Link 1 and Link 2 respectively.

Washing Step	Link File Used
Before adding Amplifier	Link 1
Before adding Label probe	Link 1
Before adding Substrate	Link 2

Table 2. ELx405 Select Washer Link File Used for QG assay.

Automated plate washing was performed using the ELx405™ Select Microplate Washer (Bio-Tek Instruments). The programming “link” function of the washer was used to link a dispense routine that dispenses 290 µl of wash buffer to one of two different wash procedures that dispenses and

aspirates 390 μ l of wash buffer. The specific parameters for each procedure are listed in Table 1 and differ only in the number of cycles programmed. A three cycle wash routine was used for all of the wash steps except the wash step after the addition of label probe incubation, prior to the addition of substrate, which uses a total of five wash cycles.

Results

Several washer parameters were adjusted to provide the best overall automated washer performance. Once the necessary settings were achieved, several experiments were performed in parallel plates. In each experiment the same cell lysate, probe mix, label probe, and probe substrate solutions were used for both the manual wash method as well as the automated wash method. Care was also taken to insure that the same pipette channel of a multichannel pipettor was used for all 8 replicate wells of a lysate volume in order to reduce any variability caused by pipetting. As demonstrated in Figure 3, the luminescent output obtained when using the ELx405 Select Microplate Washer was equivalent to that obtained when using the manual method. Each of these data points represent the mean of 24 individual well determinations located on three different non-contiguous strips on the plate.

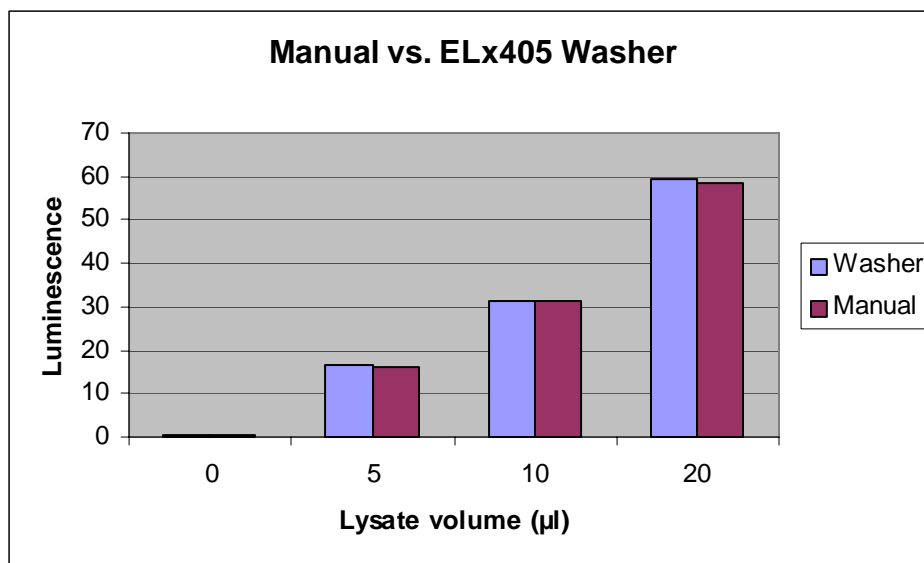


Figure 3. Comparison of Automated ELx405 Washer with manual wash method. The QuantiGene® Reagent System was used to measure GAPD levels in various volumes of cell lysates. In parallel assays, samples were prepared using either the Bio-Tek ELx405™ Microplate Washer or a manual method to perform the necessary wash steps. The data presented represents the mean of 24 determinations.

Figure 4 demonstrates the intra-assay repeatability of the QuantiGene® Reagent system when using the ELx405™ Microplate Washer. There is very close agreement of luminescent between individual wells when four different volumes of cell lysate are assayed. In this experiment a multichannel pipettor was used such that all eight wells of a strip were pipetted with the same barrel. Differences within the strip would be the result of the washer, while differences between strips is most likely the result of pipettor error. Note that while the overall signal for the 20 μ l-lysate in the last data set in Figure 4 is slightly lower than the other two corresponding data sets, the individual values are all in close agreement, with CVs of less than 4%. On average the CVs for all of the strips of the experiment were approximately 5%.

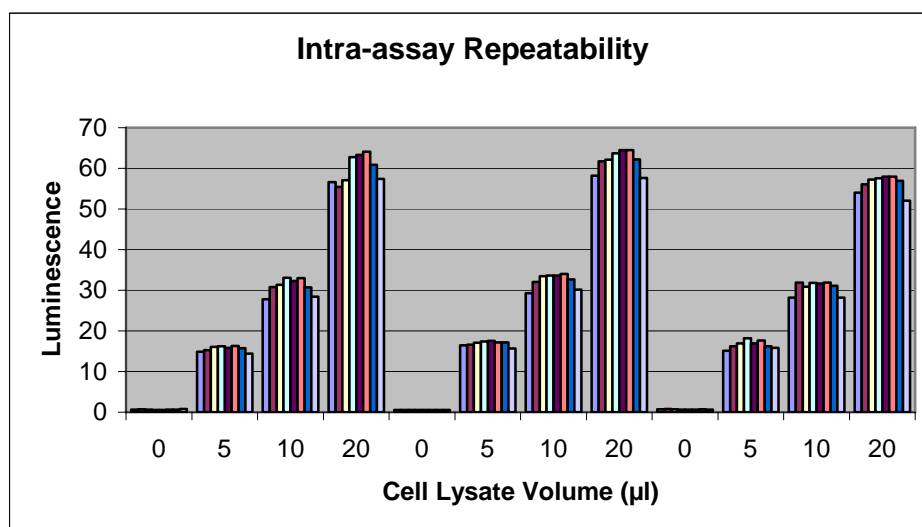


Figure 4. Intra-assay Repeatability of QuantiGene Assay using the ELx405™ Microplate Washer. Indicated volumes of cell lysates were assayed with the QuantiGene Assay System using the ELx405 to automate the wash steps. Each grouping represents a single strip of the microplate pipetted with the same barrel of a multichannel pipette, while each bar represents the result of an individual well of the microplate.

When the inter-assay repeatability was examined, the data from different experiments was observed to be consistent. As seen in Figure 5, the luminescent results from three different experiments are quite similar. The increased signal observed in experiment 1 was also observed in an assay run in parallel where the wash steps were performed manually (data not shown), which suggests that the differences observed were not the result of the ELx405™ Select Microplate Washer. Despite the differences in signal in experiment 1 from the other experiments, the relative signal was quantitative, as increased signal was consistently elevated with all cell lysate volumes tested.

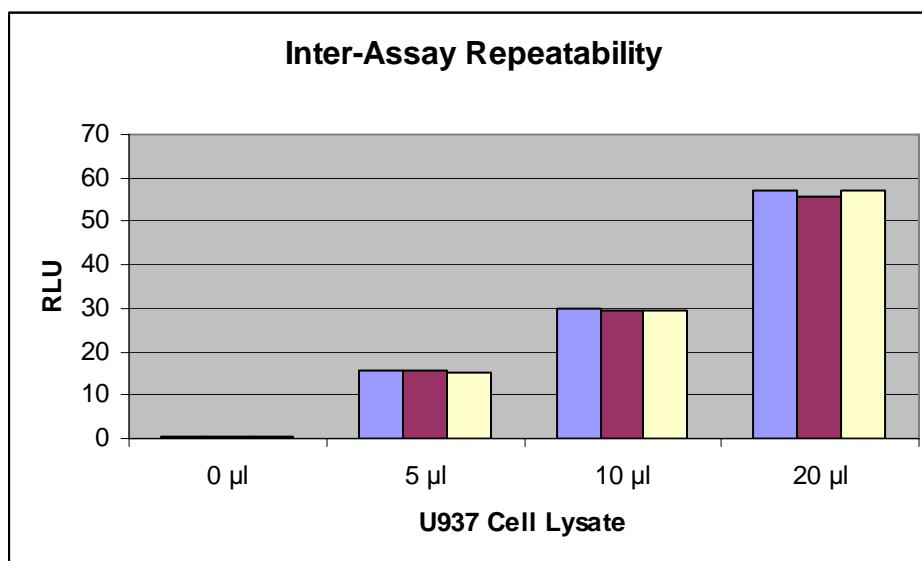


Figure 5. Inter-assay Repeatability of QuantiGene Assay using the ELx405™ Microplate Washer. Indicated volumes of cell lysates were assayed with the QuantiGene® Assay System using the ELx405 to automate the wash steps in three separate experiments performed on different days. The data for each experiment was normalized using an internal control to correct for variations in day-to-day experimental conditions. Each bar represents the mean value from 24 determinations of an individual experiment at the indicated lysates volume.

Actual Target (attomoles)	Luminescence (RLU)*	Signal - Background	Calculated Target (attomoles)	
			Polynomial	Linear
0	0.494	0	7.15×10^{-3}	0
0.04	2.17	1.676	0.0499	0.4054
0.2	8.54	8.046	0.213	0.1999
1.0	37.4	36.906	0.961	1.000
5.0	181.8	181.306	5.010	-----
25.0	609.5	609.006	24.999	-----

Table 3 Calculated vs. Expected DAP RNA target.

Figure 6 demonstrates the predictive accuracy of the QuantiGene® Reagent System when using the ELx405 to perform the wash steps. When known molar amounts (0 to 25 attomoles/well) of DAP RNA were assayed with the QuantiGene system, both the manual and the automated wash methods demonstrated a correlation with the concentration. While the luminescent raw data is slightly elevated as compared to the results obtained with a manual wash, it demonstrates very good linearity with respect to concentration. Using a 2nd order polynomial regression analysis to describe the data a very good coefficient of determination ($R^2 = 0.9988$) was obtained. The requirement for a polynomial fit is the result of the luminometer used for these studies rather than the assay. The detector in Molecular Devices Lmax Microplate Luminometer starts to become saturated at signal levels above 300, which compresses the signal from that expected. Lower concentrations (0 to 1 attomoles) demonstrate a linear correlation with the luminescent signal. When the linear equations describing the data are interpolated very close agreement between the expected concentration and the calculated concentration is observed (Table 3), indicating that the QuantiGene Reagent system in conjunction with the automated ELx405 washing procedure can reliably be used in a quantitative fashion.

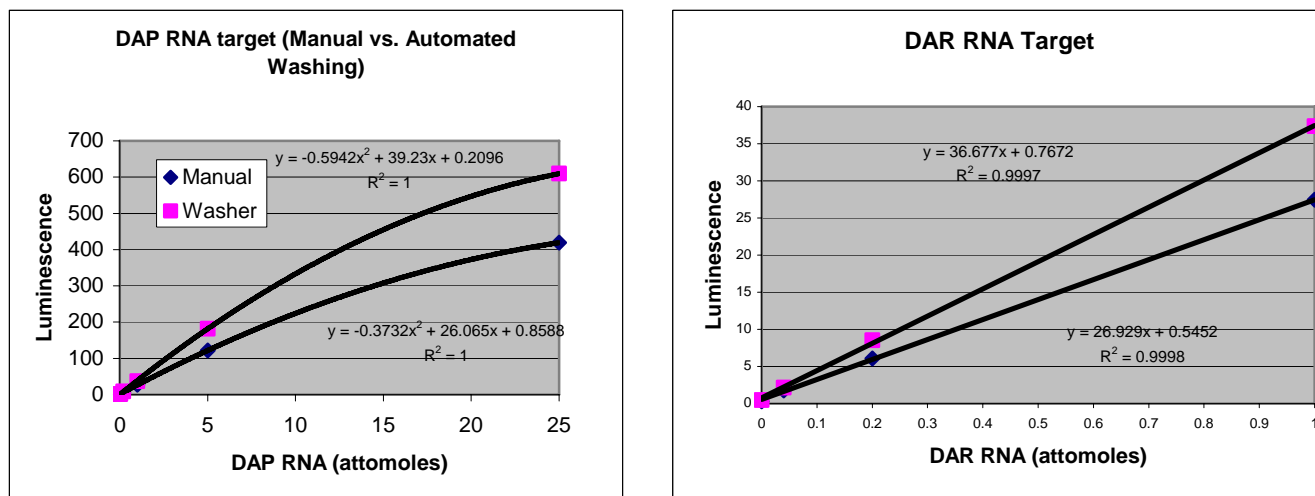


Figure 6. Comparison between manual and automated methods for washing QuantiGene assay when measuring Bacterial Control RNA (DAP). Specific amounts of target RNA were assayed using the QuantiGene® Reagent System and washed either manually or with an ELx405™ Select Automated Microplate Washer. The subsequent data was plotted and linear regression performed using Microsoft Excel. Note that each data point represents the mean of eight determinations.

When the detection limits of the QuantiGene® Reagent System are examined, both the manual and the automated wash procedure produce similar results. When the signal-to-noise ratio of the data depicted in Figure 6 is calculated for the lowest concentration measured (0.04 attomoles), a value greater than 7 is returned for the samples washed using the ELx405. Values greater than 2 are considered significantly different than the blanks. As demonstrated in Figure 7,

measurement of GAPDH from total RNA is quantitative and linear for both the manual and automated wash methods. When using total RNA as the target, this technology can detect GAPDH from as little as 18 ng of total RNA without having to amplify the target. At this level the signal to noise ratio of the measurement is greater than 25 (data not shown), which suggests that levels much lower could reliably be detected.

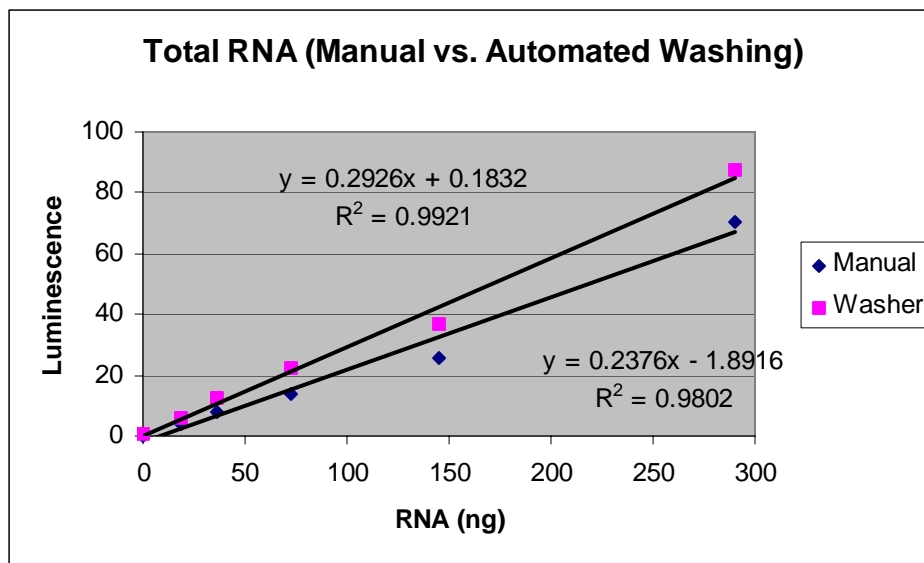


Figure 7. Total RNA Quantitation Using the QuantiGene® Reagent System washed manually or using the ELx405. RNA samples ranging from 0 to 290 ng of total RNA were assayed for GAPDH in parallel where one plate was washed manually while the other was washed using the ELx405™ Select Microplate Washer.

GAPDH expression levels in various amounts of U937 cell lysates ranging from the equivalent of 250 cells to 4000 cells were also examined. As shown in Figure 8, the QuantiGene® Reagent System provides a linear response from complex mixtures such as cell lysates. Further, the use of the ELx405 washer to automate the wash steps provides data similar to that observed when the plates are washed manually. While the raw data is slightly higher when the plate is washed using the ELx405, the ratio of signal to that of the blank is equivalent to that of the manual method (data not shown). The signal to noise ratio at the lowest cell number examined (250 cells) was calculated to be greater than 20. If one extrapolates this value in order to determine a limit of detection, theoretically lysates with less than 50 cells could be measured using this system. In addition, these data indicate that purification steps are not necessary prior to running the QuantiGene® Reagent System to specific quantitate RNA species.

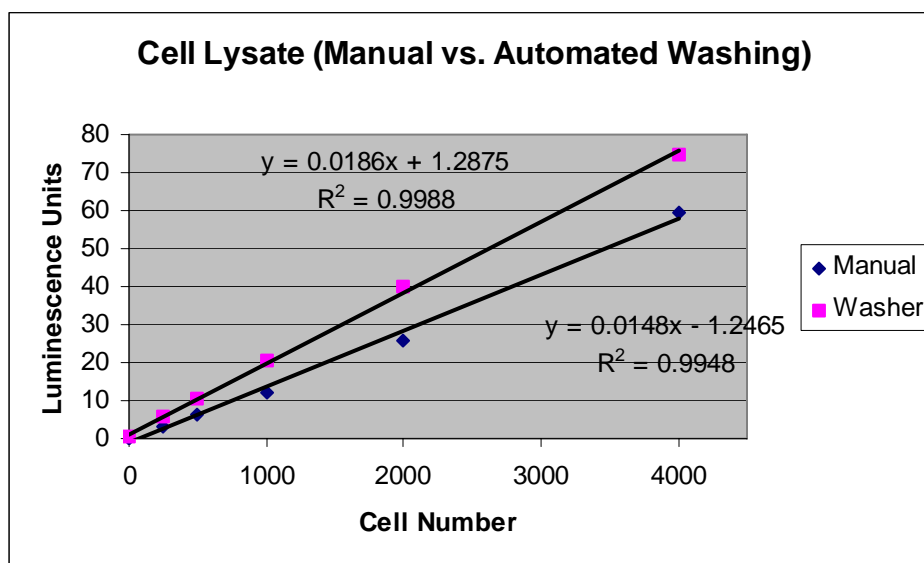


Figure 8. Comparison between manual and ELx405 automated methods of plate washing. Cell lysates calibrated to the indicated cell number were assayed for GAPDH levels in parallel using either a manual method or the ELx405™ Microplate Washer to perform the necessary plate washing steps. The subsequent data was plotted and linear regression performed using Microsoft Excel. Note that each data point represents the mean of eight determinations.

Discussion

The QuantiGene® Reagent System has been previously demonstrated to be a reliable method to accurately and precisely quantitate RNA species from a number of different matrices. However, manual washing the microplates along with the subsequent requirement of centrifugation to remove residual fluid has somewhat precluded this technology from being used to screen large numbers of samples. These data demonstrate the ability of the ELx405™ Select Microplate Washer to automate the wash procedures, as well as eliminate the need for post wash centrifugation.

In regards to the washing procedure of the assay, it was critical that all unhybridized reagents be removed after each hybridization step. Towards that end, it was observed that reducing the residual fluids after washing to be as little as possible resulted in the most reliable and repeatable results. When manually performing the assay, the microplates were inverted and centrifuged to remove the last vestiges of fluid. In order to automate the process with the ELx405 washer several parameters were adjusted to provide adequate washing and minimize residual fluids. Because the microplates used in these experiments were flat bottomed, moving the aspiration tubes towards the edge of the well reduced the liquid residual volumes significantly. This was accomplished using the horizontal offset parameter available with the ELx405 washer. The default setting of 0 places the aspiration tube in the center of the well during aspiration. Using a setting of -45 moved the aspiration tube close to the left edge of the well. The close proximity of the aspiration tube to the well edge allows for better aspiration of the residual fluid that tends to collect around the bottom rim of the well. The use of the crosswise aspiration feature can be employed to further enhance the removal of fluid. However, with the QuantiGene Reagent System the crosswise aspiration feature, while slightly decreasing the residual fluid after the final aspiration, was found to not significantly improve the luminescence results obtained and therefore was not used. Other technologies or procedure may find the crosswise aspiration effective. We also used an aspiration delay of 2 seconds (2000 msec) after the final aspiration only of the wash cycle to assist in the removal of fluid residuals. Using aspiration delays after each aspiration step was tested, but found to be of no additional benefit, while increasing the time necessary to wash the plate. In addition to decreasing the residual

volume after aspiration, increasing the number of wash cycles from that used in the manual procedure was found to dramatically improve results. The manual method relied on a centrifugation step to eliminate the residual liquid, which was particularly effective in removing unbound label probe. We found that by increasing the number of wash cycles from 3 to 5 at this last wash step decreased the background luminescent signal, while improving the well-to-well repeatability of assay. The improved results from the increase in the number of cycles can most likely be attributed to the increase in dilution from additional buffer rinses, as well as the additional agitation of non-specially bound materials.

Previous experiments had shown that preventing the aspiration tubes from touching the bottom of the well was important for having repeatable results. Allowing the aspiration tubes to travel to the bottom physically disturbed portions of the captured target that was tethered to the well bottom. Additionally, the close fitting of the well bottom to the rim of the aspiration tube would often serve as a plug, preventing the aspiration of fluid from the well. Using the aspiration depth parameter, we adjusted the aspiration tube depth such that the tubes were as low as possible without physically touching the bottom.

The ELx405™ Microplate Washer is uniquely suited to automate the wash steps of the QuantiGene® Reagent System. The 96-well manifold provides the high throughput wash capacity necessary to wash large numbers of wells. In addition, the variety of user adjustable parameters available enables the fine-tuning of the wash procedures to maximize its effectiveness. All of the adjustable parameters can be accessed by the keypad located on the instrument. Here we have described the necessary wash parameters used to perform the QuantiGene assays. While we used the ELx405 Select Microplate Washer, which is capable of washing either 96- or 384-well microplates the standard ELx405 96-well only model is also capable of performing these assays. All of the parameters altered from the default values to improve assay performance can also be accessed in this model.

Paul Held

Applications Department
BioTek Instruments Inc.

Batoul Maqsodi

Applications Department
Genospectra Inc.



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