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Application Note

Automated Washing of Magnetic Bead QuantiGene® Plex 2.0 Assays

Using the ELx405™ Magnetic Bead Washer to Wash Multiplexed mRNA Quantitation Assays

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The characterization of gene expression through the quantitation of mRNA species from experimental samples is a commonly used technique in biomedical research today. The QuantiGene® Plex 2.0 assay kit from Affymetrix offers a means to quantitate multiple mRNA species simultaneously from a variety of different sample types. This technology, which uses ferrite beads as a solid substrate for the reactions requires a magnetic field for bead retention during the wash steps. Here we describe the use of the ELx405™ Magnetic Bead Washer to automate the wash steps of this assay 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 have been developed. One such method is the QuantiGenePlex 2.0 System, which measures RNA directly from tissue homogenates, whole blood, cultured cell lysates or purified RNA without target amplification. The QuantiGene Plex 2.0 System uses branched DNA (bDNA) technology in conjunction with multi-analyte magnetic beads (xMAP® technology from Luminex Corporation) to provide the detection and quantitation of multiple mRNA targets simultaneously. The bDNA technology is a hybridization-based methodology that uses labeled DNA probes to amplify the signal rather than the target mRNA. The xMAP technology uses flow cytometry and fluorescently-dyed microspheres (beads) to allow multiplexing up to 500 unique assays within a single sample. Each hybridization step requires a thorough wash step to remove the unhybridized material. In order to increase throughput and precision of the assay, there was a desire to automate the wash steps employed by the assay.

Figure 1. ELx405 Magnetic Bead Washer

Microsphere based assays were originally designed to be used in conjunction with manual or semi-automated vacuum wash systems, such as the ELx50™ FMW [1]. While these systems worked well for low throughput demands, they do not lend themselves well to the complete automation needs of high throughput analysis. To remedy this situation and provide a platform that is readily automatable, new microsphere technologies that are partially coated with super-paramagnetic iron oxide were developed. Partial coating still allows for the red laser to illuminate the dye mixture inside the microsphere necessary for bead identification.

The ELx405™ Magnetic Bead Washer is based on the industry standard robust design of the ELx405 Microplate Washer. The ELx405 Magnetic Bead Washer offers full plate washing of magnetic microspheres. The washer uses a custom LifeSep™ magnet design from Dexter Magnetic Technologies. Two different magnets, each with high-energy neodymium iron boron magnets to rapidly separate and retain micrometer and nanometer beads, have been designed to accommodate 96- and 384-well microplate formats.

In addition to the magnetic separation capabilities, the ELx405 Magnetic Bead Washer offers all of the standard and optional features provided with the existing ELx405 washer family. As with any ELx405, the Magnetic Bead Washer accommodates standard ELISA washing with an available Dual-Action™ manifold for independent control of aspiration and dispense tubes along with an optional built-in ultrasonic cleaner for easy unattended maintenance. Automatic buffer switching for up to four wash buffers is available along with a choice of washer control using the built-in keypad or PC control via Liquid Handling Control™ Software.

Here we describe the important wash parameters of the assay and demonstrate the ability of the ELx405 to automate the wash steps employed by the QuantiGene® Plex 2.0 Reagent System.

Basis for the Assay

The QuantiGene Plex 2.0 System measures mRNA levels directly from a number of sources including, cultured cell lysates, tissue homogenates, dried blood spots, formalin fixed paraffin embedded (FFPE) sections 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 oligonucleotides with roughly half of the sequence being complimentary to sections of the target mRNA and the other half complimentary to the Capture probes (CP) immobilized onto the Capture beads. The Label Extenders (LEs) are also oligonucleotides with half of the sequence being complimentary to the target mRNA and the other half complimentary to a portion of the Pre-amplifier. The Blocking Probes (BLs) are complimentary to regions of the target mRNA not recognized by either the CE or the LE oligonucleotides and serve to reduce non-specific hybridization.

The CEs provide the assay specificity by binding the target mRNA to the capture bead. Signal amplification is the result of LEs hybridizing with the target mRNA and the Pre-Amplifier sequences. The Pre-Amplifier trunk is then hybridized with multiple Amplifiers that form the branches of bDNA. The “branches” of each amplifier have multiple biotin moieties, which will in turn bind Streptavidin-conjugated R-phycoerythrin (SAPE), the measureable signal for detection. 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 (Figure 2). The assay is designed to be compatible in batch or completely automated modes using BioTek’s ELx405 Magnetic Bead Washer.

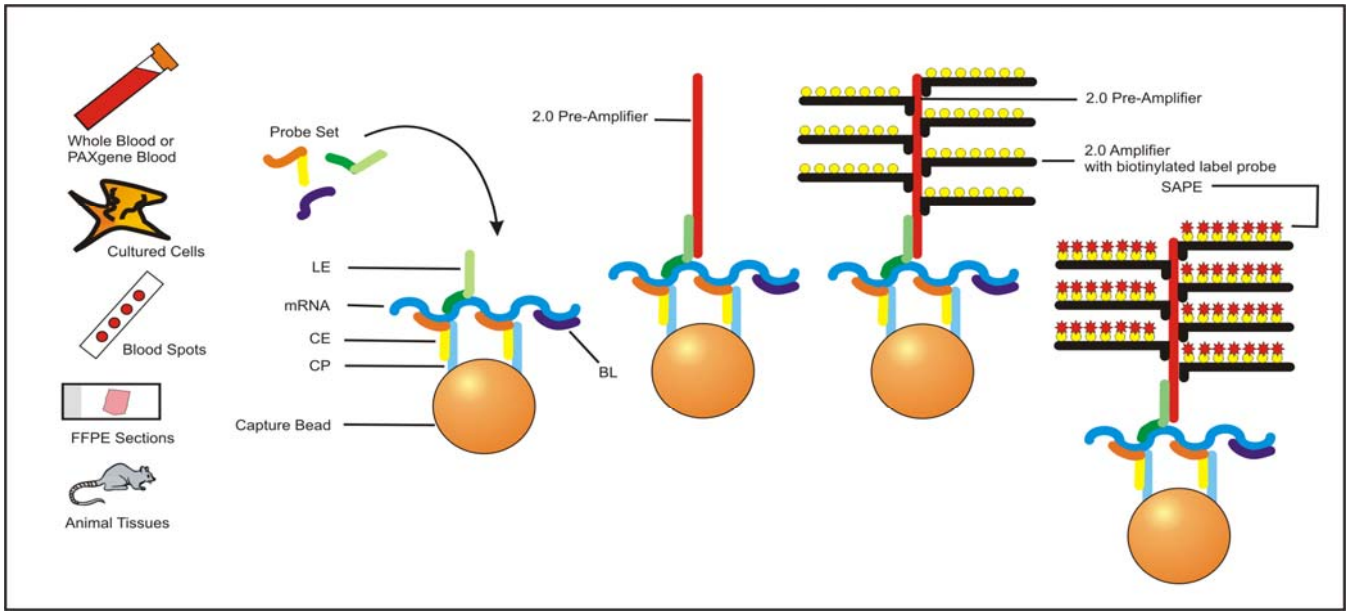

Figure 2. Overview of the QuantiGene Plex 2.0 System.

Materials and Methods

QuantiGene® Plex 2.0 Assays were performed as described in the assay kit instructions. The mRNA specific Probe Sets (heated to 95º for 5 minutes immediately prior to use), Lysis Mixture, mRNA specific Capture Beads, Proteinase K and Blocking Reagent were combined according to the kit instructions to make Working Plex set. Each well then received 20 µL of Working Plex set along with 80 µL of human total RNA and the Hybridization Plates were sealed and incubated at 54°C ±1ºC for 16-20 hours overnight in a shaking incubator at 600 rpm.

The following day, the overnight hybridization plates were briefly centrifuged. The contents were transferred to a Magnetic Separation Plate and were washed using an ELx405™ Magnetic Bead Washer (BioTek Instruments) as described in the washing instructions below. Wash Buffer consisted of 190 mL of nuclease free water, 0.6 mL of Wash Buffer Component 1, and 10 mL of Wash Buffer Component 2. After washing, 100 µL of Pre-Amplifier working solution, prepared immediately prior to use, was added to each well. The plates were resealed and incubated for 60 minutes at 50°C ± 1ºC while shaking at 600 rpm. After incubation, the plates were again washed with an ELx405 Magnetic Bead Washer followed by the addition of 100 µL of Amplifier working solution, prepared immediately prior to use, to each well. The plates were resealed and incubated for 60 minutes at 50°C ± 1ºC with shaking at 600 rpm. The plates were again washed with an ELx405 Magnetic Bead Washer and 100 µL of Label Probe working solution, prepared immediately prior to use, was added to each well. The plates were resealed and incubated for 60 minutes at 50°C ± 1ºC with shaking at 600 rpm. Following Label Probe hybridization, the beads were again washed as described, and 100 µL of SAPE working reagent was added to each well. The plates were resealed and incubated for 30 minutes at room temperature (RT) while shaking at 600 rpm. Following SAPE binding, the beads were washed as described using SAPE Wash Buffer rather than standard wash buffer. Each well then received 130 µL of SAPE Wash Buffer, placed on a shaking platform set to 600 rpm for 2-5 minutes at room temperature. Following resuspension, the samples were then read using a Luminex 100/200 reader (See Figure 3).

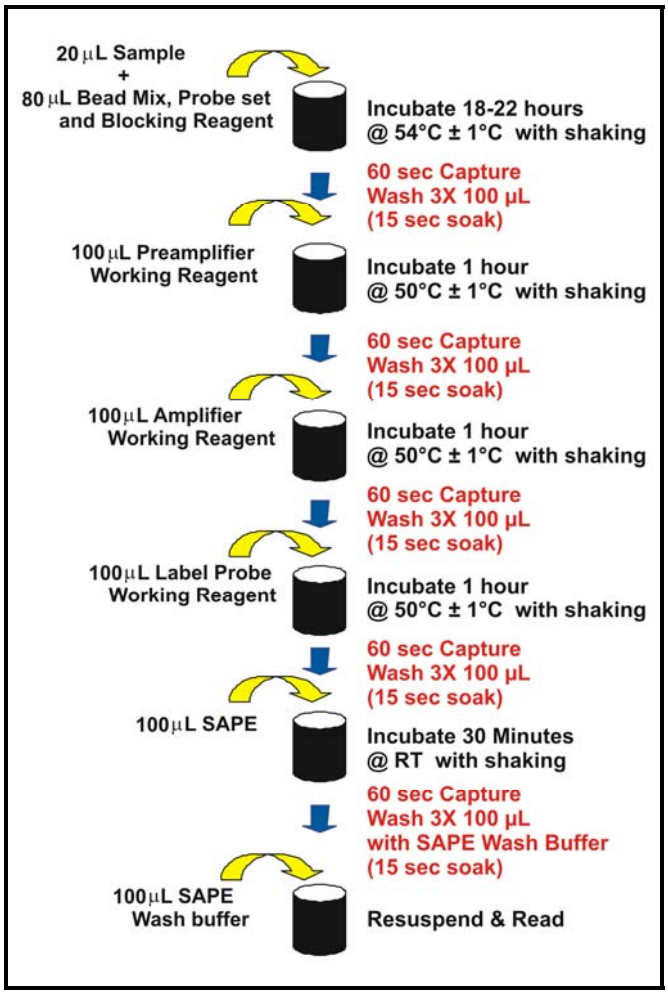

Figure 3. QuantiGene Plex 2.0 Assay Protocol using an ELx405 Select Magnetic Bead Washer. Using an initial soak time of 60 seconds to capture the beads, wash programs consisting of a series of aspiration and dispense steps each followed with a soak under magnetization were performed.

Washing Instructions

Automated plate washing was performed using the ELx405 Magnetic Bead Washer (BioTek Instruments). The programming "link" function of the washer was used to link a soak routine (Soak60) to one of two different wash procedures depending on the plate matrix. The soak routine allows for a 60 second bead capture by the magnet, while the wash procedures dispense and aspirates 100 µL or 80 µL of fluid to 96- or 384-well plates respectively. The wash routines have also utilized short soak routines between cycles to allow the recapture of any beads resuspended during the fluid dispense. The specific parameters for each procedure are listed in Table 1 and differ in the physical placement of the aspiration and dispense tubes to accommodate either 96- or 384-well microplates. These parameters have been optimized for both bead retention and washing efficiency.

Program Name	Soak60	BK96	BK384
Link File			
File Type	Soak	Wash	Wash
Method			
Wash Buffer		A	A
Plate Type		96	384
Number of Cycles		3	3
Soak/Shake		Yes	Yes
Soak Duration	60 sec	15 sec	15 sec
Shake Before Soak		No	No
Prime		No	No
Prime Volume			
Prime Flow Rate			
Dispense			
Dispense Volume		100	80
Dispense Flow Rate		1	1
Dispense Height		120	095
Horizontal Dispense Position		00	00
Horizontal Y position		00	00
Bottom Wash First		No	No
Bottom Dispense Volume			
Bottom Flow Rate			
Bottom Dispense Height			
Bottom Dispense Position			
Prime		No	No
Prime Volume			
Prime Flow Rate			
Aspiration			
Aspiration Height		48	48
Horizontal Aspiration Position		-45	00
Aspiration Rate		1	1
Aspiration Delay		00	10
Crosswise Aspirate		No	No
Crosswise Aspirate on Crosswise Height			
Crosswise Horizontal Position			
Final Aspiration		Yes	Yes
Final Aspiration Delay		0000 msec	0010 msec

Table 1. ELx405™ Magnetic Bead Washer settings for QuantiGene® Plex 2.0 Assay.

Results

These data demonstrate the ability of the ELx405 Magnetic Bead Washer to effectively perform the washes necessary for the QuantiGene Plex 2.0 Assay. In collaboration with Affymetrix, a series of QuantiGene Plex 2.0 assays were run. These hybridization-based assays use specific probe sets in order to capture and quantify specific RNA species. After the initial RNA capture, which takes place overnight, the assays uses a series of wash steps, reagent additions, and controlled temperature incubations to amplify the target and bind a fluorescent signal (Figure 3).

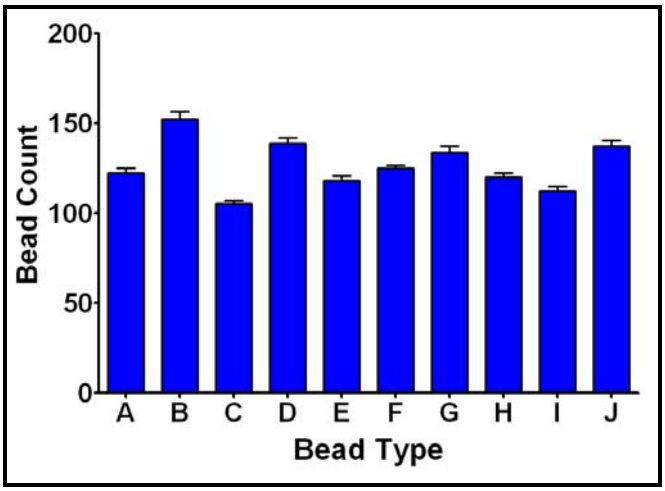

Figure 4. Average bead count of different bead types in a multiplex reaction.

Bead recovery was assessed by observing the bead count of different bead types in a 10-plex assay. After adding 1000 beads of each bead type, the beads were subjected to the same reagents, wash buffers and number of washes before being read on a Luminex reader. The Luminex reader was configured to either count at least 100 beads of each type or count for a total of 45 seconds.

As demonstrated in Figure 4, all of the different bead types from 32 different wells of a 10-plex assay averaged 106 beads or greater.

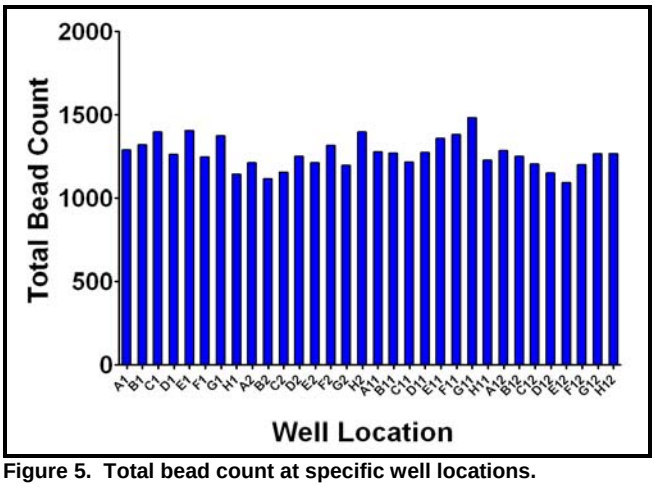

Figure 5. Total bead count at specific well locations.

In addition, for each of the individual wells all of the different bead types recorded at least 100 beads before the reader moved to the next well based on time rather than bead count (data not shown). This indicates that there were adequate numbers of beads in all of the wells after 5 separate wash procedures of 3 cycles each. The low variability in the count for each of the 10 different bead types indicates that the variability in their means is an indicator of differences in starting bead concentrations. When total bead counts from individual wells are examined an average of 1267±16 beads were counted (Figure 5). In addition, each of the individual wells had virtually the same total number of beads, which indicates that the washes performed were uniform across the entire 96-well plate.

Removal of unbound materials is a critical component in providing accurate and reliable data when using the QuantiGene® Plex Assays. In regards to the use of an automated instrument such as the ELx405™ Magnetic Bead Washer, well-to-well uniformity depends on accurate and repeatable dispense-volumes, low residual volumes and reliable bead retention during the wash steps. By using a 36-plex assay of house-keeping genes in conjunction with purified known RNA samples, the uniformity of signal response when using the ELx405 Magnetic Bead Washer to wash the assay in 96-well microplates can be examined. As demonstrated in Figure 6, the fluorescent signal of an RNA species is consistent across the entire plate

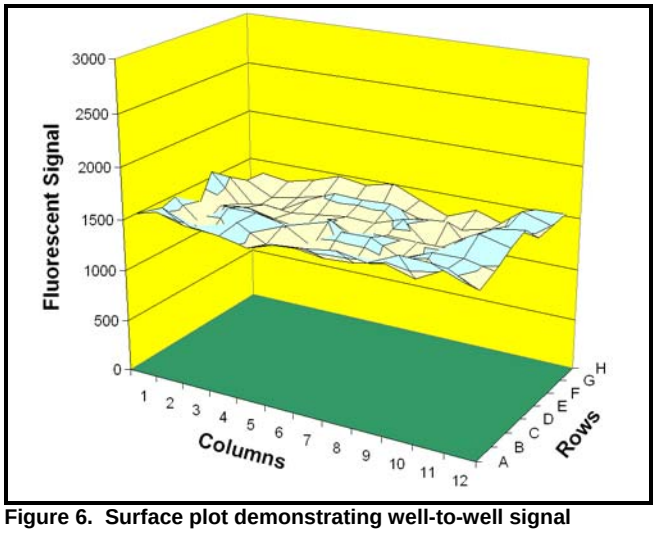

Figure 6. Surface plot demonstrating well-to-well signal uniformity. NFKB1 RNA was measured from purified RNA aliquoted into all of the wells of a 96-well microplate as part of a 36-plex QuantiGene Plex assay. The fluorescent signal was then plotted using Microsoft Excel.

When other unrelated RNA species are examined from the same 36-plex assay similar results are observed. Table 2 indicates the mean value and %CV of the fluorescent signal returned when the same RNA pool was aliquoted into all the wells of a 96-well plate. By design the copy number of these different RNA species was intended to be similar to each other to minimize assay artifacts. On average the %CV was 9.2% for all 36 RNA targets, which is significantly lower than the typical ≥ 15% CV found in many vacuum based wash procedures.

#	Target	Mean	%CV
1	ABCB4	819	7.68
2	NFKB1	1459	7.86
3	RELA	1196	8.63
4	IFNG	1041	7.40
5	TNF	967	11.37
6	UGT1A8	917	8.64
7	UGT1A9	1520	9.34
8	ABCB11	1540	8.66
9	ACTB	1250	9.12
10	IL1B	2533	9.69
11	TNFRSF6	373	8.12
12	BAD	1285	9.27
13	CYP2B6	1253	7.82
14	CSF2	1494	10.32
15	TNFSF6	3235	9.98
16	IL8	262	13.07
17	PPIB	1021	8.59
18	VEGF	2166	12.05
19	ABCC2	1309	8.55
20	IL10	1624	8.90
21	GAPDH	1604	9.20
22	ABCB1	1664	9.45
23	CYP3A4	511	9.59
24	SLC22A7	1825	8.89
25	CYP2A6	981	9.21
26	CYP1A2	1358	10.75
27	IL2	806	8.05
28	IL6R	1501	8.49
29	PTK2B	968	9.06
30	CDKN1A	680	9.06
31	UGT1A4	1248	9.92
32	CHUK	644	9.70
33	BCL2	962	8.02
34	UGT2B10	796	7.43
35	UBT2B7	773	11.60
36	UGT2B4	494	8.62

Table 2. QuantiGene Plex 2.0 signal uniformity using the ELx405 Select Magnetic Bead Washer. A 36-plex assay was performed using total human RNA. Data represent the mean and %CV of 96 determinations.

When different samples are quantified in either 96- or 384-well microplates very consistent and similar results are observed. Figure 7 compares the levels of 10 different RNA species in two different human total RNA samples using a 10-plex QuantiGene Plex 2.0 assay. Each data point is the mean of four different replicates of the sample. In all RNA species significant differences in RNA levels could be distinguished between the two RNA sources. In addition, the blank wells (no RNA) had very low signal (<10) relative to the experimental samples indicating that the determined signal was not an artifact of poor washing.

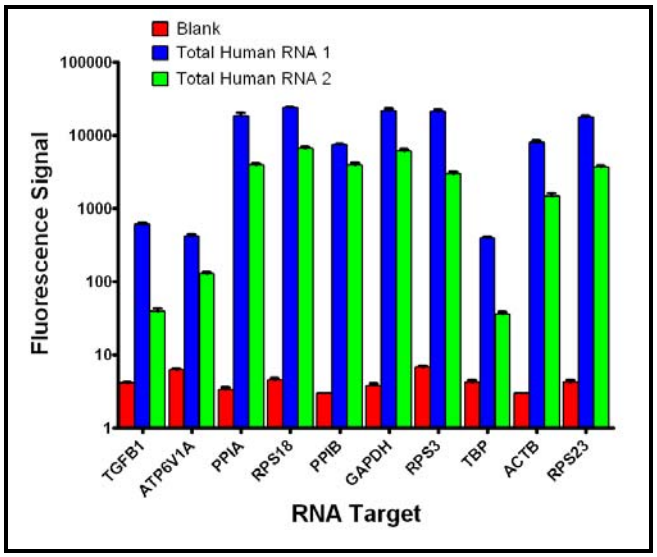

Figure 7. Expression of RNA species in different RNA samples using 96-well reaction plates. A 10-plex QuantiGene® Plex 2.0 assay was performed on human total RNA and compared to the levels in a human RNA sample. Data represent the mean of 4 replicates.

A more difficult challenge is to perform wash steps in 384-well microplates. The demand to reduce sample size in order to decrease reagent usage has provided the impetus to higher density plate formats. However, the smaller well size makes the aspiration of fluids more difficult in regards to bead retention. By increasing the height of the aspiration tubes relative the bottom of the well, adequate washing was obtained without significant loss of the beads.

Figure 8 demonstrates the ability of the ELx405 Magnetic Bead Washer to wash magnetic beads in a 384-well format. Again, comparing two different human total RNA pools, four different samples were taken from each pool and assayed in 4 replicates each. Despite the difficulty in washing and retaining beads, the blank wells remained low, while the experimental wells had orders of magnitude greater signal. In addition, replicate samples had very similar results. Assay precision, as measured by %CV, using the magnetic washer was less than 15% in the 384-well format.

Discussion

The last ten years has seen the growth of suspension arrays, notably the Luminex xMAP® technology. This technology allows for flexibility in biomolecular analysis, as well as allowing multiplexing analytes in a single reaction. This serves to significantly reduce the cost inherent to analysis. Further, the reaction kinetics is similar to solution-based chemistries which are a distinct advantage relative to planar, chip arrays. Products for genotyping, gene expression and protein applications are available from over 30 Luminex reagent partners. Working with Affymetrix, BioTek has developed a magnetic bead washer capable of automating and improving the precision of magnetic bead-based gene expression assays, but the technology can be used to improve multiplexed assays similarly across the application space defined by the Luminex partners.

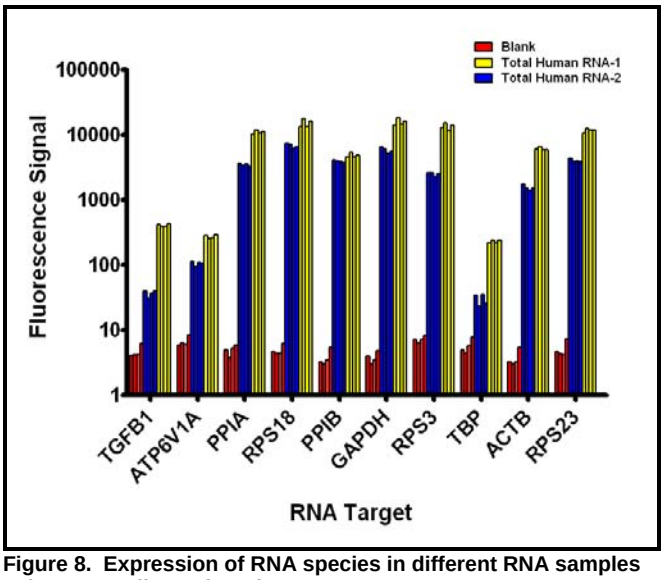

Figure 8. Expression of RNA species in different RNA samples using 384-well reaction plates.

The custom LifeSep™ magnet plate from Dexter Magnetic Technologies supplied with the ELx405 Magnetic Bead Washer provides advantages over many off the shelf magnets. The two different magnets have each been designed for 96-well or 384-well applications. The 96-well version uses 8 magnet bars that travel through the center of 12 wells. In other words each row (A-H) has a separate magnet. Likewise the 384-well version has 24 magnetic bars that travel through the centers of each column of a plate. Because the magnet does not encompass the entire bottom of the well, the aspiration tubes of the washer can be offset away from the beads preventing them from being lost via aspiration. The common magnet also reduces the variability between different magnets that can be associated with 96 different magnets. The magnets are also removable for easy cleaning and safe storage when not in use. Their strong magnetic field (6,800 Gauss for 96-well, and 4,300 Gauss for 384-well also provides for rapid bead separation.

References

1. Held, P. Using the ELx50 Filter Microplate Washer to Perform the Wash Processing Steps Required for Luminex xMAP Assays. BioTek Application note, www.biotek.com.

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