An Evaluation of Two Commercial Hybridisation Technologies

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Abstract

It is well-established that one of the major drawbacks of traditional spotted microarray technologies is that there is very little mixing of solution under a coverslip and thus the signal obtained from any one spot tends to be the result of hybridisation to only a small proportion of the potential targets in solution. Several different methods can be used to improve target hybridisation through increased mixing. A common design for dealing with this limitation involves sealing the hybridisation solution into a closed system and using a rotisserie-like set-up. An air bubble introduced into the system facilitates mixing of the solution during rotation. While this simple method is relatively effective and proven, it does have some limitations: generally high volumes of liquid are required, and trapped bubbles can cause portions of the array to dry out. One novel method to enhance mixing involves the use of acoustic waves to pulse the hybridization solution across the array surface. Here we compare the two methods by using Agilent microarrays with the Agilent SureHyb Oven and the Advalytix Slide Booster. Our results indicate that the acoustic wave mixing of the Advalytix device enhances signal, lowers noise and reduces the necessary volumes for hybridisation.

Introduction

The conceived uses for microarrays continue to expand well past the original application to gene expression. As their utility grows so does the need to perfect the technology in order to generate the most accurate results possible. The mechanics of the hybridisation step continues to be one of the stumbling points of this technology, regardless of its application.

The original method of hybridisation was that of simple diffusion. A labelled sample was held against an array by a coverslip or a second array in a sandwich. This unit was then placed in a sealed humidified chamber over night. The greatest flaw in this system was that it was dependent on diffusion alone. It has been suggested that by this method, only about 0.3% of the labelled sample is capable of binding to the arrayed probes.[1] This is due the fact that the probe is only capable of a diffusion coefficient of about 10^{-6} to 10^{-7} cm^2/s such that a single strand of labelled probe will only move on the order of a millimetre during a 24 hour hybridisation.[2]

Another method of hybridisation is to use a rotisserie similar to what is has been used for northern and Southern blotting for years. By inserting an air bubble into the system the hybridisation solution flows across the surface of the array within a closed system. The potential pitfalls of this system are inherent to premise on which it is based. The air bubble inserted into the system may become “stuck” and cause sections of the array to dry out and be exposed to little to no probe. Furthermore, the system generally involves a significantly larger amount of solution (often 4 to 10 times more) thereby diluting probe, again creating unfavourable kinetics.

Currently there is a large amount of research into microfluidics and devices that can be used to better generate an even distribution of the probe across the array in the minimal amount of time thereby allowing the system to reach an equilibrium state at a faster rate.[2,3] One commercially available apparatus which provides both temperature control and mixing is the Slide Booster from Advalytix. This system uses surface acoustic wave micro-agitation to efficiently mix small volumes typical of those used in basic diffusion setups.[4] An additional advantage of this system is that it does not require any moving parts or pumps that can lead to failures over time.

Here we compare the rotisserie technology of the Agilent SureHyb System and the micro-agitation design of the Advalytix Slide Booster in an attempt to determine which method provides the better results in terms of spot intensity and signal to noise.

Methods

Total RNA from HeLa cells and commercially prepared Universal Human Reference RNA (Stratagene) were amplified using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent). Half of the samples were hybridised in the SureHyb Oven (Agilent) and the hybridisation mix was made following the 60-mer oligo microarray processing protocol (SSPE wash) designed for the SureHyb System (Agilent). The remaining samples, were hybridised on the Slide Booster (Advalytix), and required a smaller total volume of hybridisation mix. For this reason, the mix was scaled down such that all relative proportions remained intact (see Table 1).

Table 1: Hybridisation Mix components and volumes for each method.

The arrays hybridised in the SureHyb oven were assembled in SureHyb hybridisation chambers following the manufacturer’s protocol. Briefly, the hybridisation mix was added to a gasket slide place in the base of a SureHyb chamber. The array was placed on top and the chamber was reassembled. The arrays were incubated for 17 hours at 65°C with a rotation speed of 4 rpm. The remaining arrays were hybridised following manufacturer’s protocols in the Slide Booster. Briefly, 20 µl of Advason™ coupling buffer was placed on top of each agitation chip and the humidifying chambers were filled with 500 µl of humidifying buffer. Arrays were placed on top of the agitation chips and the chambers were brought up to 65°C before the hybridisation mix was loaded underneath a Liftersip™ (Erie Scientific) and left to incubate for 17 hours with agitation. All arrays were washed together following the SSPE wash protocol (Agilent). Slides were scanned in the Agilent G2464AA DNA Microarray Scanner and quantified using Agilent Feature Extraction v8.0. A total of 10 arrays were used.

Component	Volume for SureHyb (µl)	Volume for Slide Booster (µl)
Sample (1ug/channel) + nuclease-free water	165	38
10x Control Targets	50	10
25x Fragmentation Buffer	9	2
2x Hybridisation Buffer	225	50
Total Volume	449	100

Results and Discussion

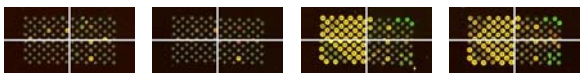

Figure 1: Images of the Four Corners. The QC reports from the Feature Extraction software provide images depicting the four corners of the arrays with crosses marking the centres. As can be seen here, the arrays hybridised on the Slide Booster (251239128861 & 251239128862) have visibly brighter spots than those arrays hybridised in the SureHyb oven (251239128859 & 251239128860)

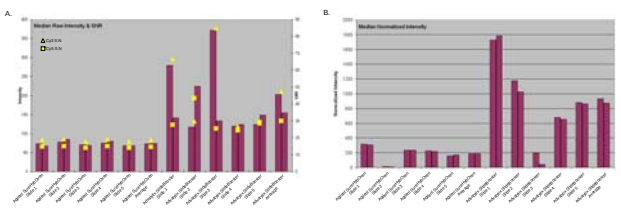

Figure 2: Median Intensity Levels. The median intensity levels were for each array as well as for the average across all of the arrays hybridised by each method. This is shown for both the raw intensities and the signal to noise (A) and the normalised intensities (B). It is apparent that the Slide Booster generates higher signal intensities but often with a pronounced dye disparity. When looking at the normalized intensities the dye disparity is lost as would be expected, however, it is also quite obvious that 1 array in each category has significantly lower intensity levels which were not apparent in the raw data.

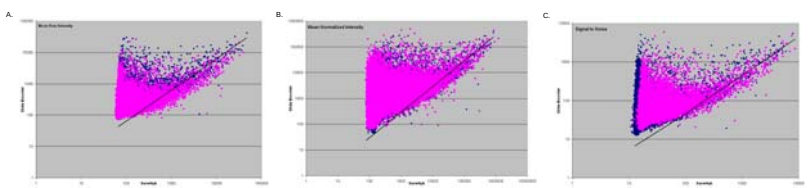

Figure 3: Intensity and Signal to Noise Scatter Plots. The average across all of the arrays for each method, the raw intensity (A), normalized intensity (B) and signal to noise (C) were plotted for each spot and each channel. An overall shift compared to the 1:1 line was expected in the direction of the better technology. However, what is seen is the shift is primarily located at the low end of the SureHyb spectrum while the high end spots remain evenly distributed around the 1:1 line.

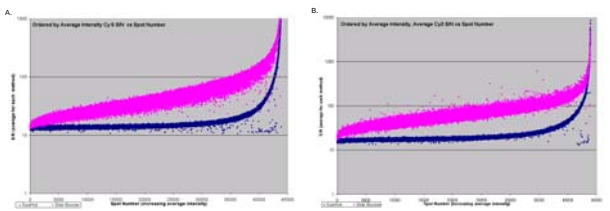

Figure 4: Average Signal to Noise as ordered by Average Intensity. The signal to noise was ordered by the average intensity and then plotted against the spot number which is representative of the increasing average intensity. This is seen for the Cy5 channel (A) and the Cy3 channel (B). The levels of signal to noise from the SureHyb system are consistently at the same level regardless of the intensity change until the brightest intensity spots at which point there is a dramatic jump in signal to noise implying that the noise is increasing at the same rate as the intensity for much of the range or else there is very little change in the intensity across the majority of the array. Whereas the levels of signal to noise from the Slide Booster increase more dramatically with the increase of intensity implying that the noise levels are much more consistent across the array and is independent of the spot intensity and that there is a better dynamic range.

In an attempt to discern which hybridisation method provided better results, the arrays were first compared visually. Figure 1 clearly demonstrates that more spots are present on the arrays hybridised on the SlideBooster than on the SureHyb system. What is presented are the four corners of the arrays. While the visual analysis indicated a general increase in signal intensities, examination of the extracted data was still required. To address this, the median spot intensities for each array and the average across all the arrays used for this method was then plotted with the signal-to-background ratios (Figure 2). As can be seen, both the raw intensity values and the normalised values show a distinction between the two hybridisation methods. The median signal to noise values were also plotted with the raw intensities and show a similar trend. One array from each method shows remarkably low normalised intensities in comparison to the rest of their groups, something that is not present in the raw levels. One potentially important observation is that the arrays hybridised on the SlideBooster all have a large dye bias typically toward Cy3. However the trend is not always consistent and other experiments with the SlideBooster have not show this effect thus we do not think that it can be said that the system favours one dye over the other. We are uncertain at this point if the effect may attributed to the now well documented problems with ozone mediated degredation of Cy5 and related dyes.

The average intensity per spot on the Agilent SureHyb processed arrays was plotted against the average intensity per spot on the Advalytix SlideBooster processed arrays (Figure 3). In such a plot, if both methods provided similar results the plot should be evenly distributed around the 1:1 line. What is seen however is that there is a definite shift towards the SlideBooster, especially for the spots at the low end of the intensity range. Instead of a consistent general shift away from the 1:1 line there was a dramatic shift for spots that were of low intensity on the Agilent SureHyb system, indicating that for some reason these two systems are in strong disagreement at these spots. Without confirming the results using qPCR it is difficult to determine at this point which of these results are the most accurately representative of the gene expression within the biological system. Further investigation will help to determine if this issue is widespread over the array or if there are localised regions that are showing decreased intensity on the SureHyb system as might be expected if the bubble stopped rotating over these arrays.

Increases in signal to noise ratios are generally seen as a benefit, however looking at global averages for such trends does not provide detail as to whether this is affecting the low or high intensity signals primarily or if this is a generalised trend. The average signal to noise levels for each spot ordered by the average intensity for the same spot and then plotted for each channel and each hybridisation method (Figure 4). As can be seen, the signal to noise levels for the SureHyb system are consistent regardless of what the intensity level is, implying that the noise levels remain proportional to the intensity levels. In contrast, the arrays hybridised in the SlideBooster show an increase in signal to noise with an increase of intensity implying that noise levels are not proportional to the intensity levels. This indicates that the noise produced by the SlideBooster is much more evenly distributed across the arrays and is not dependent on the spot intensity.

The mechanics of diffusion have proven to be too slow for any given probe to cross the entire length of an array in a reasonable period of time and the ability to create randomly distributed replicate spots on the array surface is mechanically difficult when creating homemade arrays. For these reasons there have been many papers describing the need for mixing within the hybridisation step of microarray analysis as well as the description of technologies created for this purpose.[2,7] Mixing increases the ability of each labelled probe to reach its target within a workable period of time.[2,7] Thus a more accurate representation of the gene expression of the samples is attained.

An ideal method of mixing would allow for the advantages created by having a low sample volume, and thus a higher concentration of labelled probe, as well as having flexibility within the conditions such that different types of arrays, different types of hybridisation buffers, and different types of environmental conditions can be used in the same system. The SureHyb system takes into account some of these requirements by allowing temperature control and rotation speed control. However, it is a system that requires that specific backing slides and hybridisation chambers to be used even if the type of array and buffer are flexible. The SlideBooster system allows for greater flexibility as different humidifying buffers can be used depending on the chemistry of the hybridisation buffer and there is no restrictions put upon the type of coverslip, in fact this system also allows for sandwiching two arrays. Again all environmental factors are flexible and under the users control and several different such conditions can be used on the same device at the same time (up to four different combinations of temperature, time, mixing intensity etc…).

The Advalytix Slide Booster demonstrated higher intensity levels, both pre- and post-processing, than the Agilent SureHyb System’s Hybridisation Oven. The same was true for the signal to noise ratios. At this time however, more work needs to be done to test reproducibility and accuracy of the results. What is particularly important is a more in depth determination of the apparent dye bias issues as well as an external validation that the increased signal intensities seen on the Advalytix system are truly representative of actual biological conditions. While these questions still need to be evaluated, it is clear that the Advalytix SlideBooster system presents a potentially novel and superior hybridisation technology that can benefit users of Agilent microarrays.

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