

## CodeLink™ Bioarray Image Threshold Flagging Procedure When Using the Agilent G2565BA Scanner



Signal strength was found to be different on the Agilent G2565BA scanner from that on the Molecular Devices GenePix™ 4000B scanner, making it necessary to adjust the threshold accordingly. It was determined that a signal strength of 1.25 on the G2565BA scanner is equivalent to a signal strength of 1.00 on the GenePix 4000B scanner.

### Introduction

CodeLink™ Bioarrays from Applied Microarrays, Inc. (“AMI”) can be processed using different scanners because of the standard 1 x 3-in microscope slide format. Currently, the most widely used platforms with the CodeLink™ arrays are the GenePix 4000B (Molecular Devices) and G2565BA (Agilent Technologies) scanners. The CodeLink™ Bioarray platform has been optimized during product development on the GenePix 4000B scanner, and image quantitation flags assigned by the software are tailored for this scanner type. However, with the widespread use of the G2465BA scanner, there is an immediate need for assessment of quality flags and optimization of image flagging thresholds on these images to gain consistency between the two scanners. This application note discusses the differences between data obtained on the GenePix 4000B and G2565BA scanners for CodeLink™ Whole Genome Bioarrays and provides a procedure for setting the appropriate thresholds when using a G2565BA scanner.

### Materials

#### Products Used

CodeLink™ Human Whole Genome Bioarray, six-pack	300026-6PK
CodeLink™ Expression Analysis Software Kit v4.1	310033-00
Cy™5-Streptavidin	PA45001

### Other Materials Required

G2565BA microarray scanner (Agilent Technologies)

GenePix 4000B Microarray Scanner (Molecular Devices)

GenePix Pro 4.0 Microarray Image Analysis Software (Molecular Devices)

Scanner Calibration Slide, DS01 (Full Moon Biosystems)

### Methods

In this study, 40 CodeLink™ Human Whole Genome Bioarrays were processed using standard protocols and scanned on both the GenePix 4000B scanner and the G2565BA scanner. These arrays were processed with Cy5-Streptavidin and images were captured at a wavelength of 635 nm. For the GenePix 4000B scanner, the PMT gain was 600V with 100% laser power and 5-µm pixel size. For the G2565BA scanner, the PMT was set at 70% with 100% laser power and 5-µm pixel size. This PMT setting for the G2565BA scanner was optimized using the DS01 calibration slide (1).

### Results and Discussion

The CodeLink™ Bioarray images from both scanners were analyzed and differences identified for the automated threshold flags assigned during image processing. Signal strength was calculated by the CodeLink™ v4.1 software for each probe on the array using each spot’s local background and signal value. Signal strength is defined as signal mean divided by background median plus 1.5 standard deviations of local background. Therefore, the threshold is determined locally around each spot and offers a finer resolution than a fixed global threshold.

$$\text{Signal strength} = \frac{\text{Signal mean}}{\text{Background median} + 1.5 \text{ standard Deviations of background}}$$

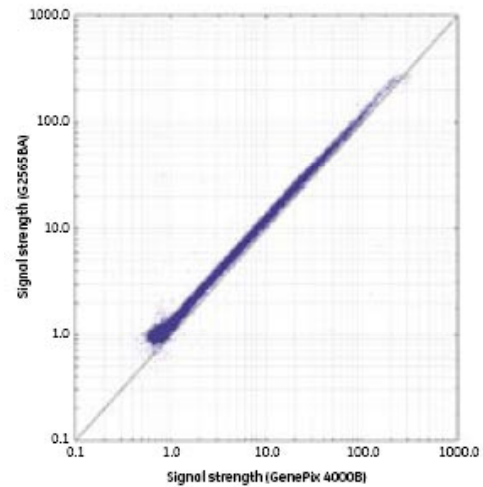
CodeLink™ Bioarrays surpass the product performance specifications on both the G2565BA and GenePix 4000B scanners, yet there was a discrepancy in the signal strengths between these two scanners using the setting in the current protocol (1). The signal strength for each probe was higher on the G2565BA scanner relative to the GenePix 4000B (Fig. 1).

This difference is important for threshold flagging (i.e. “L” or “G”) because these assignments are made using the signal-strength values. Because the CodeLink™ flagging thresholds were tailored using the GenePix 4000B scanner, this difference in signal strength on the G2565BA scanner needs to be accounted for and thresholds adjusted accordingly.

Figure 2 provides an example of the CodeLink™ software v4.1 output, exported to Microsoft Excel™ format, to illustrate the relationship between signal strength and quality flags. The values in the signal strength column are used by the CodeLink™ software to determine which values are above and below noise for each probe. When the signal strength is below 1.00 for the GenePix 4000B scanner, the probe is considered below threshold and is assigned the flag “L”, which can be found in the Quality\_flag column.

Figure 3 provides a close-up view of the data points near threshold. The data points in Figure 3 are plotted in linear space and fit to a linear regression model. The linear-fit equation illustrates the relative difference of signal-strength measurements between the GenePix 4000B and G2565BA scanners. Using this formula, it was determined that a signal strength of 1.00 on the GenePix 4000B scanner is equivalent to 1.25 on the G2565BA scanner. In addition, the unadjusted signal strength for negative control probes on G2565BA and GenePix 4000B scanned images is illustrated in Figure 4, which further justifies the necessary shift to a 1.25 signal-strength threshold for the G2565BA scanner. Therefore, to set the threshold flags to equivalency, the signal-strength threshold on the G2565BA scanner needs to be adjusted to 1.25.

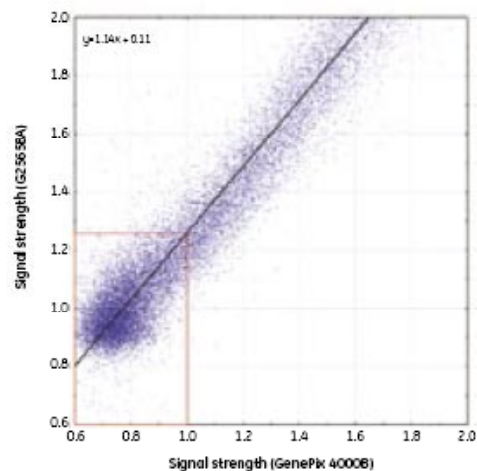
The improvement in the flagging assignment between the GenePix 4000B and G2565BA scanners is illustrated by the decreased number of green data points from Figure 5 to Figure 6, comparing non-adjusted with adjusted values.



**Fig. 1** Probe signal-strength distribution for GenePix 4000B vs G2565BA scanner. The entire distribution of signal strengths was higher on the G2565BA than on the GenePix 4000B.

	N	O	P	Q
11	Raw_intensity	Normalized_intensity	Quality_flag	Signal_strength
12	15590.2549	117.3442	G	141.5207
13	1099.6952	8.2771	G	11.8798
14	-0.5306	-0.004	L	0.8307
15	28.26	0.2127	G	1.1304
16	5.9821	0.0441	L	0.8905
17	24.1	0.1914	G	1.0922
18	20.3051	0.1528	G	1.063
19	9890.9248	74.4467	G	97.6069
20	164.4615	1.2379	G	2.3012

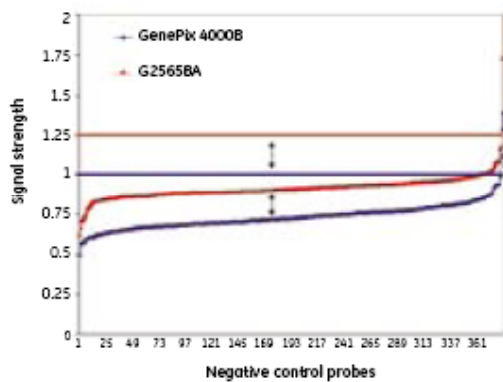
**Fig. 2.** Example output from CodeLink™ software v4.1. This output example demonstrates the relationship of signal strength to quality flags. Values below 1.0 in signal strength (column Q) for the GenePix 4000B scanner are flagged as “L” in the Quality\_flag column (column P).



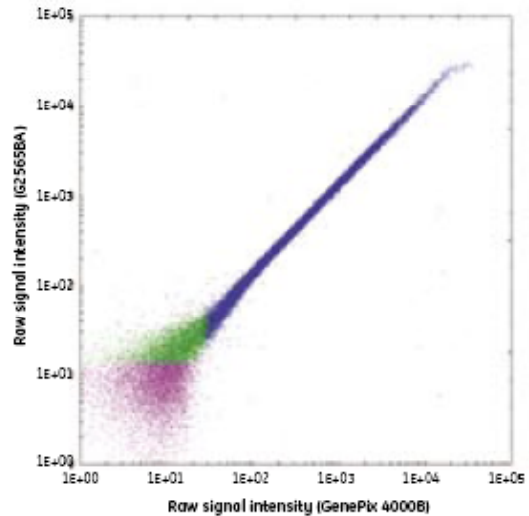
**Fig. 3.** Probe signal-strength distribution at the level of noise for the GenePix 4000B vs the G2565BA scanner. All probes exhibiting signal strength between 0.6 and 2.0 are plotted. The best linear regression fit,  $y = 1.14x + 0.11$ , is displayed as the black line through the center of the distribution.

Improvements in the flag correlation, using the new threshold for the G2565BA scanner, can be seen in the Venn diagrams of Figure 7. Figure 7A shows the concordance in “L” flags between the GenePix 4000B and G2565BA scanners using the current thresholds (1.00) for both scanners. The union represents the probes that are below threshold (“L”) for both scanners. The nonunion represents the number of probes that are above threshold on the indicated scanner while below on the other scanner. In this case, 7482 probes were above noise (“G”) on the G2565BA scanner while below noise on the GenePix 4000B scanner.

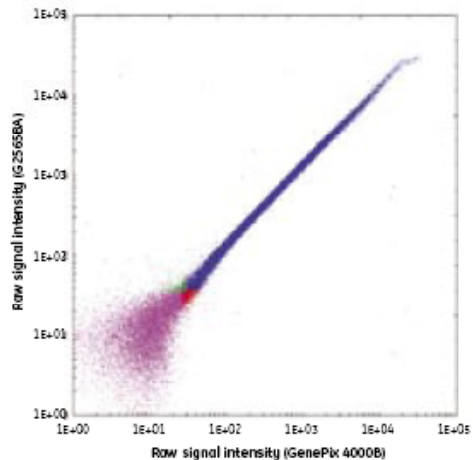
Figure 7B shows concordance in “L” flags between the GenePix 4000B and G2565BA scanners using the adjusted signal-strength threshold for the G2565BA scanner. The signal-strength threshold used in this Venn diagram was 1.00 for the GenePix 4000B scanner and 1.25 for the G2565BA scanner. In this case, 469 probes were above noise on the G2565BA while below noise on the GenePix 4000B scanner; conversely, 911 probes were above noise on the GenePix 4000B and below noise on the G2565BA scanner.



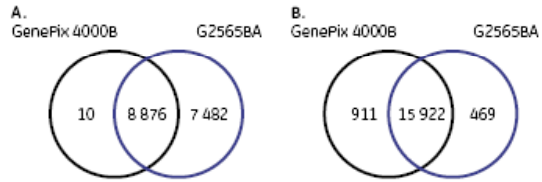
**Fig. 4.** Signal-strength distribution for negative control probes using the GenePix 4000B and G2565BA scanners. In this graph the distribution of negative-control probe signal strengths is plotted for both the unadjusted G2565BA scanner (red) and the GenePix 4000B scanner (blue). The horizontal lines represent the suggested signal-strength threshold settings (1.00 for GenePix 4000B and 1.25 for G2565BA).



**Fig. 5.** Distribution of raw signal intensities for the GenePix 4000B vs the G2565BA scanner with unadjusted flags illustrated. Blue data points represent signals that are above threshold on both scanners; pink = below threshold on both scanners; green = above threshold on the G2565BA but below threshold on the GenePix 4000B.



**Fig. 6.** Distribution of raw signal intensities for the GenePix 4000B vs the G2565BA scanner with adjusted flags illustrated. Blue data points represent signals that are above threshold on both scanners; pink = below threshold on both scanners; green = above threshold on the G2565BA but below threshold on the GenePix 4000B; red = above threshold on the GenePix 4000B but below threshold on the G2565BA.



**Fig. 7.** Venn diagrams showing the level of concordance for probes flagged below noise between the GenePix 4000B and G2565BA scanners. A: Concordance in “L” flags between the GenePix 4000B and G2565BA using the current threshold of 1.00 for both scanners. B: Concordance in “L” flags between the GenePix 4000B and G2565BA using the adjusted signal-strength threshold of 1.25 for the G2565BA.

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## Conclusions

Because the signal strength has been found to be different on the Agilent G2565BA scanner from that on the Molecular Devices GenePix 4000B scanner, it is necessary to adjust the threshold accordingly. It was determined that a signal strength of 1.25 on the G2565BA scanner is equivalent to a signal strength of 1.00 on the GenePix 4000B scanner.

## Appendix: Reassigning “L” Flags When Using the Agilent G2565BA Scanner

1. Output the image data into .txt file format from the CodeLink software v4.1 or 5.0 by choosing **File > Export Analysis Results > Custom Output > .txt file.**
2. Ensure that the .txt file output contains the necessary fields for reassigning the flagging threshold. If this is your first time exporting data from the software, open one of the .txt files within Microsoft Excel and view the column headers (Fig. 2).
3. When using the G2565BA scanner, assign all probes with signal strength less than 1.25 an “L” flag while retaining all the other flag assignments.

## References

1. Application note: Scanning CodeLink Bioarrays on Agilent G2565BA microarray scanner, GE Healthcare, 63-0050-80, Edition AC (2004).