

## Rinse and Post-scan Re-rinse Protocol for Background Improvement



CodeLink™ Expression Bioarray System is a high performance gene expression system that includes high quality bioarrays (pre-arrayed oligonucleotide slides), reagents, optimized protocols, parallel processing kits and instrumentation, analysis software, and full product support.

The CodeLink™ Expression Bioarray User's Guide describes the procedure for processing bioarrays. The current protocol incorporates a new final rinse protocol using a single, 30-s rinse in the buffered solution of 0.1xSSC/0.05% Tween™20 rather than using a 0.05% Tween solution in two 5-s rinses. This application note presents data that demonstrates the improvement obtained with this new rinse protocol, while verifying the back-compatibility of data produced by the new method.

This application note also describes the use of the new rinse protocol for post-scan re-rinsing as a suggested method for eliminating certain high background contaminants that can be visible during scanning. The previous rinse protocol did not recommend that bioarrays undergo re-rinsing due to the potential for non-uniform and significant signal loss. However, the new method, which can be used as a re-rinse or in lieu of the final rinse step, offers several benefits. First, it allows for re-rinsing to remove background artifacts with minimal and uniform spot intensity loss. Second, as the new rinse solution is a buffered solution, the protocol can reduce lab-to-lab variations in buffer pH brought about by varying water quality and/or environmental conditions. Finally, it simplifies the protocol, providing a single 30-s rinse rather than two 5-s rinses.

### Products Used

As the CodeLink™ Expression Bioarray User's Guide comprises the processing procedure for all bioarrays in the CodeLink™ Expression series thus far, this post-scan re-rinse protocol and modified post-hybridization final rinse can be applied to all bioarray products in the series. These include:

CodeLink™ Human Whole Genome Bioarray	300026-6PK
CodeLink™ Rat Whole Genome Bioarray	300031-6PK
CodeLink™ Mouse Whole Genome Bioarray	300033-6PK
CodeLink™ Human Inflammation v1 16-Assay Bioarray	300076-3PK
CodeLink™ Rat Inflammation v1 16-Assay Bioarray	300077-3PK
CodeLink™ Mouse Inflammation v1 16-Assay Bioarray	300072-3PK
CodeLink™ Human Inflammation v1 16-Assay Bioarray	300076-1PK
CodeLink™ Rat Inflammation v1 16-Assay Bioarray	300077-1PK
CodeLink™ Mouse Inflammation v1 16-Assay Bioarray	300072-1PK
CodeLink™ Human ADME v1 16-Assay Bioarray	300081-3PK
CodeLink™ Human ADME v1 16-Assay Bioarray	300081-1PK

## Protocol

### 1. Preparation

The protocol will produce one liter of 0.1xSSC/0.05% Tween 20 buffer for slide rinsing or re-rinsing. All reagents used should be molecular biology grade. The user should prepare sufficient buffer volume for the entire rinsing or re-rinsing procedure. Approximately 250 ml of 0.1xSSC/0.05% Tween buffer is needed for 12 slides.

1.1 In a clean 1-liter container, add the following:

994.5 ml deionized water  
0.5 ml Tween 20  
5 ml 20x SSC buffer (Ambion Catalog No. 9763)

1.2 Mix well by swirling. This solution can be stored up to two weeks at ambient temperature.

### 2. Rinsing

After scanning CodeLink™ Expression Bioarrays, slides can be re-rinsed using the following steps.

2.1 Place slides into a clean bioarray rack and place the rack into a large reagent reservoir completely filled with 0.1x SSC/0.05% Tween 20 at ambient temperature. Incubate the slides for 30 seconds with mild agitation up-and-down.

2.2 Follow process steps 6.8-6.11 in the CodeLink™ System Processing section of the CodeLink™ Expression Bioarray System User Guide.

2.3 Follow process steps in Section 7 in the same section of the User Guide with one possible modification: If the original scan of a given slide is needed, add “\_rerinse” after the slide number to allow use of the slide msr file.

## Methods

**Experimental Method 1 (see Table 1 and Figure 2):** Mouse testes total RNA was labeled and amplified per the CodeLink™ standard procedure. The positive control mRNA spikes for sensitivity determination were at an estimated mass ratio of 1:300 000. Preparations were assayed on four independent CodeLink™ UniSet™ Mouse I bioarrays. Assays were performed with Cy™5-Streptavidin and analyzed with CodeLink™ Expressions Analysis v2.3 software.

**Experimental Method 2 (see Figure 1 and Figure 3):** Target was prepared from human brain total RNA. The positive control mRNA spikes for sensitivity determination were at an estimated mass ratio of 1:300 000. Preparations were assayed on four independent CodeLink™ UniSet™ Human I bioarrays. Assays were performed with Cy™5-Streptavidin and analyzed with CodeLink™ Expression Analysis v2.3 software.

**Experimental Method 3 (see Figure 4):** Target was prepared from human liver total RNA, assayed with Cy5-Streptavidin, and analyzed with CodeLink™ Expression Analysis v2.3 software.

Final Rinse	Re-rinse	Average	Minimum detectable fold-change sensitivity	
		%CV	% ratios within two-fold	% positive controls above threshold
0.05% Tween, 2x5 s	No re-rinse	9.44	99.7	99.5
	Re-rinse with 0.1xSSC/0.05% Tween, 1x30 s	9.97	99.8	99.4
0.1xSSC/0.05% Tween, 1x30 s	No re-rinse	10.65	99.7	99.8
	Re-rinse with 0.1xSSC/0.05% Tween, 1x30 s	9.15	99.7	99.5

**Table 1.** A high degree of reproducibility in assay performance (see Experimental Method 1) is obtained using either the original final rinse buffer or the new final rinse buffer.

### Assay Performance Metrics

A battery of microarray experiments was performed using the new rinse protocol for the final rinse during standard processing and/or as a method for post-scan re-rinsing. Reproducibility, sensitivity, and a baseline response comparison were then assessed for the new solution and the original solution of 0.05% Tween buffer. In addition, results obtained from bioarrays rinsed initially with 0.1xSSC/0.05% Tween buffer were correlated with results from bioarrays rinsed in the original 0.05% Tween buffer, and to slides re-rinsed with 0.1xSSC/0.05% Tween.

### Reproducibility

Minimizing process-induced variation is essential to obtaining accurate and reproducible data from microarray experiments. Within- and between-preparation reproducibility can be measured by the coefficient of variation (CV) or by determining the minimum detectable fold-change between experiments. Low variability in within-condition experimental comparisons results in more accurate determinations of expression level changes in between-condition experimental comparisons.

Table 1 demonstrated that spot signal CVs were highly reproducible when re-rinsing post-scan with the new protocol, irrespective of the rinse protocol used initially. Total CVs were 9.97% and 9.15% with the original or the new buffer rinse as initial rinse, respectively. In addition, replacing the final rinse of the post-hybridization protocol with the new rinse buffer had little impact on the current high level of reproducibility of spot signals; bioarray experiments using the original buffer led to a total CV of 9.44%, whereas experiments using the new buffer led to a similar total CV of 10.65%. In addition, bioarray results from all final rinse conditions showed a high percentage of probes with signal intensities within a two-fold range. The minimum detectable fold-change was two-fold for over 99% of the probes when using the re-rinse procedure regardless of which buffer was used in the post-hybridization final rinse step. Therefore, the assay reproducibility is comparable whether re-rinsing post-scan slides or replacing the post-hybridization final rinse step with the new rinse buffer.

### Sensitivity

To assess the sensitivity of the CodeLink™ microarray platform when using different buffers, spiking experiments were conducted using positive control mRNAs at known mass ratios (see Experimental Method 1). These spikes were used to determine the level of signal detectable above a threshold or local background. Sensitivity was measured either as the percentage of signals above threshold or as signal-to-threshold (S/T) ratios. These parameters can be calculated for positive control spike probes as well as for discovery probes across the slide.

Table 1 demonstrates that the sensitivity of assays using the re-rinse procedure is comparable to the sensitivity typically obtained with the original final rinse buffer. The percentage of positive control signals above threshold at an estimated mass ratio of 1:300,000 (based on a 5% mRNA content assumption) does not vary significantly after re-rinsing the slides regardless of the rinse buffer used in the post-hybridization final rinse step. Percentages above 90% are typically observed with the current protocol at this mass ratio, varying somewhat depending on target preparation and RNA source tissue. For the experiment described in Table 1 (see Experimental Method 1), the S/T ratio results for the positive control probes were not statistically different after re-rinsing the slides using the re-rinse procedure (data not shown).

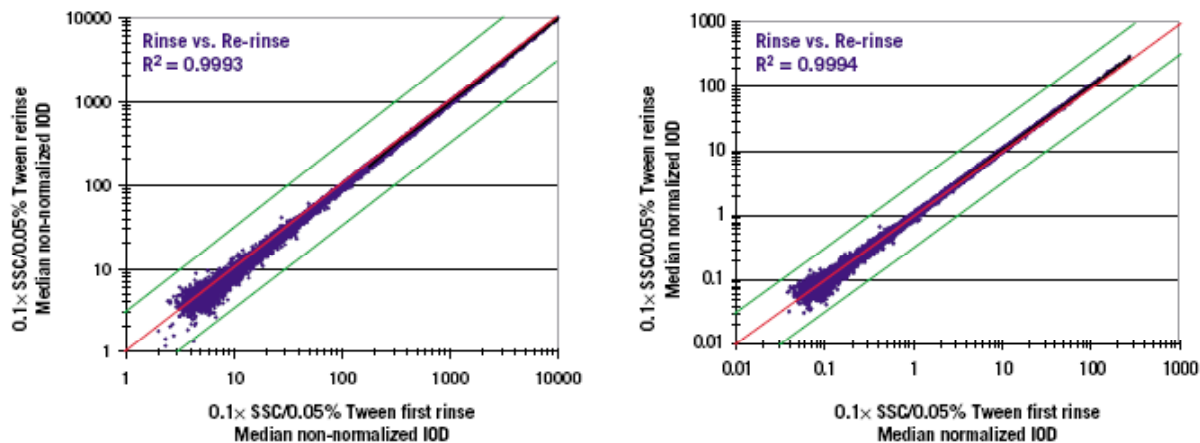
#### **Data Compatibility Between the Original Post-Hybridization Final Rinse Procedure, the New Final Rinse Procedure, and the Re-Rinse Procedure**

Correlations of probe intensities obtained from bioarrays run using the re-rinse procedure and the original post-hybridization final rinse protocol are shown as log/log plots in Figures 1 and 2. Figure 1 compares 0.1xSSC/0.05% Tween post-hybridization final rinse to 0.1xSSC/0.05% Tween re-rinse, and Figure 2 compares 0.05% Tween post-hybridization final rinse (original) to 0.1xSSC/0.05% Tween re-rinse. The figures present both normalized and non-normalized data, and confirm that post-scan re-rinsing did not alter the signals obtained outside of normal assay variation. Data obtained with either the original or new final rinse buffers followed by a re-rinse procedure produced similar probe median normalized intensities across all discovery probes in the CodeLink™ expression assay. In fact, these data demonstrate a linear relationship between conditions with a correlation coefficient of 0.999 or greater. Greater than 99.4% of probe intensities between all of these conditions were within two-fold. Non-normalized signal data also correlated well to the normalized data, again supporting the conclusion that this re-rinse procedure does not significantly alter the experimental results obtained from CodeLink™ gene expression assays. The variation introduced by re-rinsing post-scan or by using the new rinse buffer in the post-hybridization final rinse is similar to normal variation between slides processed within the same buffer.

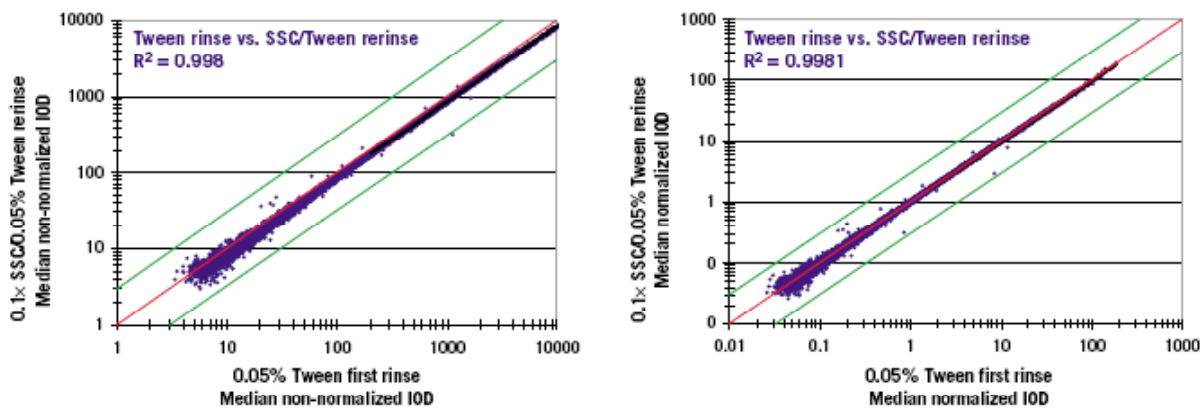
Similarly, when probe intensities obtained from experiments using the original rinse were correlated to those obtained with the new rinse buffer, good correlation in data were observed, indicating that the new rinse buffer is a reasonable substitute for the current buffer. Figure 3 shows comparisons using both normalized and non-normalized data for each rinse type. Data obtained with either the original or new final rinse buffers produced similar probe median normalized intensities across all discovery probes in the CodeLink™ expression assay, showing a linear relationship between conditions with a correlation coefficient (R) of 0.9963 or greater. Greater than 99.5% of probe intensities lie within two-fold across these two conditions. Non-normalized signal data correlated well to the normalized data, again supporting the conclusion that this new rinse procedure does not significantly alter the experimental results obtained from CodeLink™ gene expression assays. Figures 1, 2 and 3 support the conclusion that the variations introduced by re-rinsing post-scan or by using the new rinse buffer in the post-hybridization final rinse is similar to normal variation between slides processed within the same buffer.

In separate experiments, results of bioarray data using the new rinse buffer in place of the original Tween buffer were compared to a performance baseline for the product formed over multiple experiments and slide batches. No significant performance outliers were observed from this experiment, indicating that no back-compatibility issues should be observed when using 0.1xSSC/0.05% Tween buffer as the post-hybridization final rinse solution in place of the original 0.05% Tween buffer (data not shown).

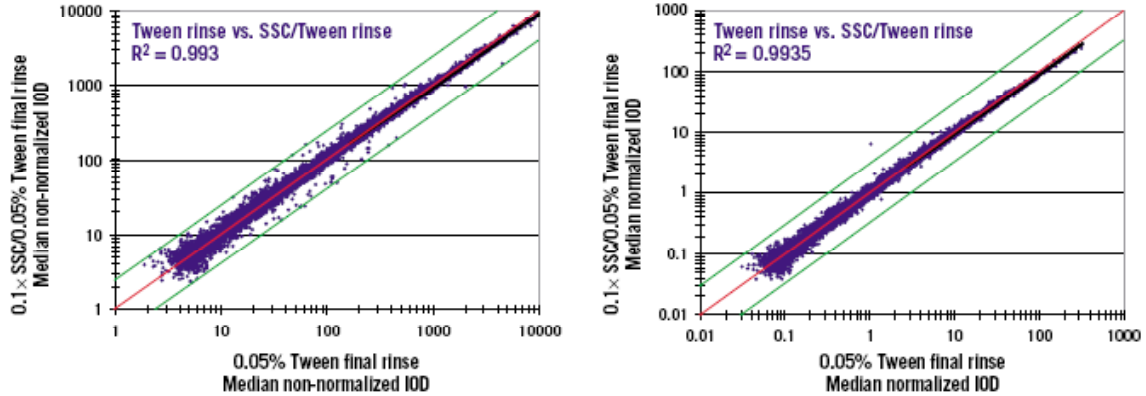
Although not all streaks or other artifacts on bioarrays slides will be removed by this method, the new buffer allows re-rinsing to be attempted without significant loss of signal. Figure 4 compares two bioarray images pre- and post-rinse to demonstrate the effectiveness of the suggested re-rinse procedure. On the left, a drying artifact has caused streaks across the slide. The image on the right shows the same slide after the new re-rinse protocol; the artifact has been removed with a linear loss of a minimal amount of signal intensity. As seen in Figures 1 and 2, initial rinse versus re-rinsed slides correlate strongly with either the original or new buffer as initial rinse solution.



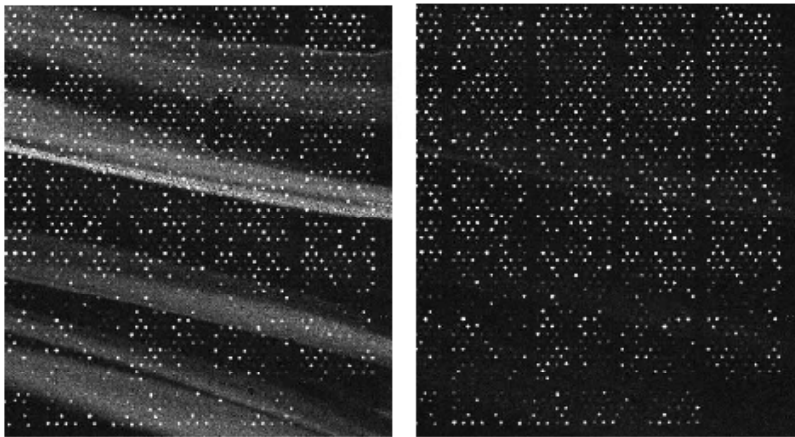
**Fig 1.** Log/log plots of discovery probe median intensities for SSC/Tween rinse and SSC/Tween re-rinse (non-normalized at left; normalized at right) reveal good correlation between data obtained following each rinse method (see Experimental Method 2). The green lines indicate two-fold changes in intensity.



**Fig 2.** Log/log plots of discovery probe median intensities for Tween first rinse and SSC/Tween re-rinse (non-normalized at left; normalized at right) reveal good correlation between data obtained following each rinse method (see Experimental Method 1). The green lines indicate two-fold changes in intensity.



**Fig 3.** Log/log plots of discovery probe median intensities for original Tween rinse vs. new SSC/ Tween rinse (non-normalized at left; normalized at right) reveal good correlation of data obtained following each rinse type (see Experimental Method 2). The green lines indicate two-fold changes in intensity.



**Fig. 4.** A drying artifact (streak in left slide) is removed using re-rinse solution 0.1xSSC/0.05% Tween for a single rinse of 30 s (slide at right shows image obtained after re-rinse). The data demonstrated a linear loss of a minimal amount of signal intensity, as observed in the other experiments in these studies (see Experimental Method 3 and Fig. 1 and 2).

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