Reuse of cDNA microarrays hybridized with cRNA by stripping with RNase H

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DNA microarrays are powerful tools for global analysis of gene transcript expression. However, their high cost and the need for replication have limited their use. Here, we report a new stripping technique applicable to microarrays hybridized with cRNA with RNase H that is reproducible, leaving the DNA oligonucleotide probes intact and available for adding two additional uses. A Pearson correlation was used to assess the agreement between the first-round hybridization and the second- and third-round hybridizations. Significant correlations (R^2*, 0.9893 and 0.975;* $P < 0.001$*) were observed among virgin arrays and stripped arrays hybridized with the same sample. Additionally, statistical class comparison analysis globally indicated that there were essentially no differences detected following three hybridizations. Dye-swapped microarrays produced similar results. However, arrays stripped with RNase H exhibited decreased efficiency of hybridization signal with increasing use. In the present study, the oligonucleotide microarrays can be used three times.*

Microarray analysis is a powerful tool for high-throughput, global transcriptional profiling of gene expression enabling a comprehensive view of the transcriptional response induced by a physiologic stimulus or drug treatment and allowing for the identification of sets of genes that are potentially related to its mechanism of action (1). However, the high initial cost of a microarray and the need for replication to achieve meaningful statistical significance have limited their introduction into many laboratories. Addressing this problem, Hu et al. (2) have developed an alkaline-based stripping technique to whole human genome microarrays (Agilent Technologies, Santa Clara, CA, USA) hybridized with cRNA and demonstrated that long oligonucleotide DNA microarrays can be stripped and reliably reused once. Bao et al. (3) have described a stripping protocol with a higher concentration of formamide and high temperature to strip off the probe without causing damage to the microarray. In addition, Hahnke et al. (4) have devised a method using alkali hydrolysis followed by RNase and proteinase K degradation. Here, we report a stripping technique using RNase H alone that yields a high level of reproducibility with up to three uses, thereby reducing the cost per microarray. In addition, it is performed under milder conditions and requires only two steps to perform.

RNase H is an endonuclease that cleaves the 3′-O-P-bond of RNA in a DNA/RNA duplex to produce 3′-hydroxyl and 5′-phosphate terminated products (5,6). Specifically, it degrades RNA in RNA/DNA hybrids and will not degrade DNA or single-stranded RNA. It is readily denatured at 55°C and is inactive in hybridization solutions, which contain no divalent cations.

Human umbilical vein endothelial cells (HUVEC) were obtained from Cascade Biologics (Portland, OR, USA) and cultured according to the supplier's recommendations in medium 200 (Cascade Biologics). Total RNA was isolated from HUVEC using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's protocol. Total RNA samples were run on a LabChip Bioanalyzer 2100 (Agilent Technologies) to ensure RNA quality. RNA amplification and labeling were performed following the Low RNA Input Linear Amplification Kit Plus (LRILAKP; Agilent Technologies) protocol. Human universal reference RNA (Stratagene, LaJolla, CA, USA) was used as cohybridant for each microarray. Labeled cRNA was purified using RNeasy mini-spin columns (Qiagen, Valencia, CA, USA) and eluted with nuclease-free water from the column in 30 μL final volume. Yields of cRNA were determined using NanoDrop ND-1000 UV-VIS

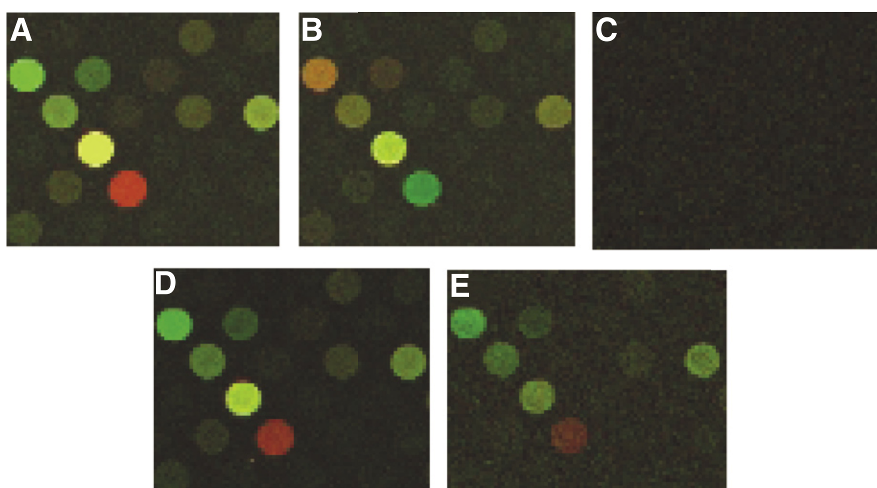

Figure 1. Images of the same section of each microarray from the sample experiments. (A) First use hybridized with cRNA (first round). (B) The same section from the dye-swapped replicate. (C) The same section from the stripped array before the second hybridization. (D) The same section from stripped array hybridized with cRNA (second round). (E) The same section from third round hybridized array (third round).

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Table 1. Average Background

Reuse Level	Background (average)			
	Red Channel	SD	Green Channel	SD
1st Hybridization	108.85	5.24	74.79	5.99
2nd Hybridization	105.64	5.95	68.74	5.33
3rd Hybridization	109.27	7.35	67.23	6.25

Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The same pool of cRNA was used for initial and subsequent hybridizations to first-use and stripped microarrays.

Following fragmentation of cRNA, whole human genome oligonucleotide microarrays were hybridized with 750 ng Cy-5 labeled HUVEC and Cy-3 labeled human universal reference cRNA at 65°C. The same RNA preparations were run on three microarrays resulting in three technical replicates. Dye-swap replicates were conducted in the first hybridization. The arrays were incubated overnight then washed once for 1 min in Gene Expression Wash Buffer 1 (Agilent Technologies) and then washed for 1 min in Gene Expression Wash Buffer 2 (Agilent Technologies).

Five hundred microliters of stripping buffer containing 100 U RNase H (Epicentre Biotechnologies, Madison, WI, USA) were made up in first strand buffer and added onto each of the used arrays. The arrays were incubated with rotation at 4 rpm at 37°C overnight. At the end of the RNA degradation, RNase H was inactivated at 55°C for 30 min, then transferred to Agilent Wash Solution 1 for 1 min and then Agilent Wash Solution 2 for 1 min. Finally, the slides were washed with water for 1 min and allowed to air-dry at room temperature. The stripped slides were stored in the dark until used again.

All microarrays were scanned under the same photomultiplier settings at 10 µM resolution using the GenePix 4000B Scanner (Molecular Devices, Sunnyvale, CA, USA) operating under GenePix 6.0 (Molecular Devices) software. GenePix results (GPR) files were analyzed using BRB ArrayTools developed by Dr. Richard Simon and Amy Peng Lam (linus.nci.nih.gov/BRB-ArrayTools.html). To evaluate microarray reproducibility, a Pearson's correlation coefficient between replicate arrays was calculated with BRB-Array Tools. The Pearson's correlation coefficient is a measure of the strength and direction of a linear relationship between two variables (7). A high, positive Pearson's correlation coefficient will be obtained if two arrays produce similar gene expression profiles.

To evaluate the quality of RNase H stripped microarrays, the same section of each microarray from the sample experiments was scanned (Figure 1). After the RNase H stripping protocol, the bound cRNA appeared to be washed off completely as shown in Figure 1C. The gene expression profile across virgin versus stripped arrays was similar and without significant increase in background (Figure 1, A, D, and E).

The log ratio of each point was calculated based on intensities between the two channels and then averaged for genes in each group. A Pearson's correlation was calculated to assess the agreement between the first-round hybridization group and the dye-swapped group in six virgin arrays, the first-round hybridization and the second-round hybridization in the same array, and the first-round hybridization and the third-round hybridization in the same array. A dye-swap experiment is usually performed to determine if a dye bias has been introduced, in which the dye-swaps are technical replicates of the same two RNA samples. All the arrays were hybridized with the same cRNA from HUVEC and Human Universal Reference RNA. The result of a Pearson's correlation calculation showed a high correlation among the first-round hybridizations (R^2, 0.970; $P < 0.001$) and third-round hybridizations in the same arrays (R^2, 0.989 and 0.975; $P < 0.001$) as shown in Figure 2. Reused arrays had similar background intensities as the first hybridization

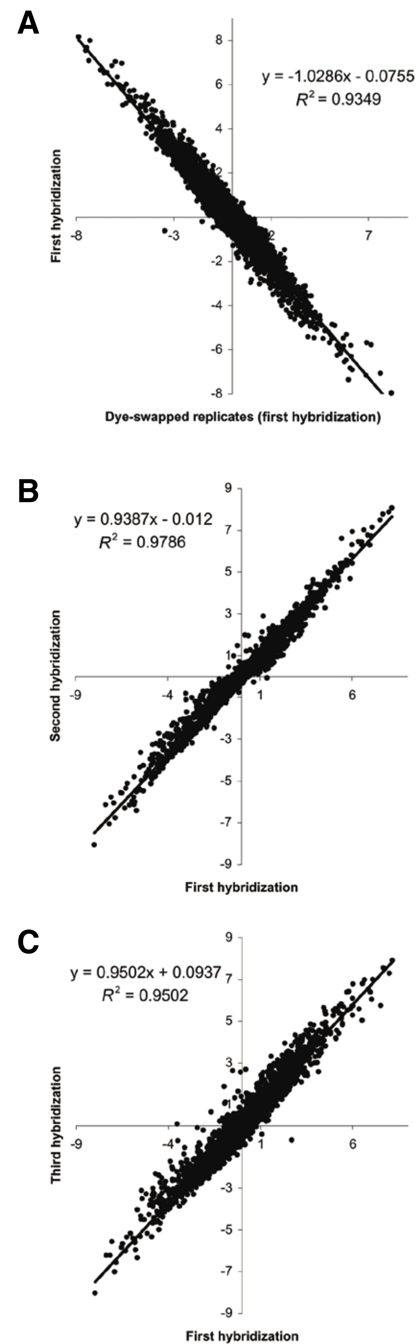

Figure 2. Scatter plots showing the correlation between the results of two different hybridizations with the same cRNA from human umbilical vein endothelial cells (HUVEC) and Human Universal Reference RNA. Log ratio of each point was calculated and averaged. (A) The dye-swapped first-round hybridizations in two groups with triplicates. A significant correlation was observed between results of the dye-swapped first-round hybridizations (R^2, 0.970; $P < 0.001$). (B) The first- and second-round hybridization in the same array. A significant correlation was observed between results of the first- and second-round hybridization (R^2, 0.989; $P < 0.001$). (C) The first- and third-round hybridization in the same array (R^2, 0.975; $P < 0.001$).

Table 2. Ratio Profiles of Some Specific Genes

Gene Names	Ratio Average-1st	SD-1st	Ratio Average-2nd	SD-2nd	Ratio Average-3rd	SD-3rd
THC2270208	0.16	0.02	0.15	0.01	0.14	0.02
HIST1H2BL	0.08	0.01	0.08	0.00	0.08	0.00
PRIM1	0.21	0.00	0.22	0.01	0.21	0.01
NUDT21	0.71	0.02	0.74	0.03	0.73	0.05
DVL1	0.73	0.03	0.72	0.02	0.71	0.01
U2AF1	0.82	0.04	0.79	0.04	0.68	0.04
PTX1	1.13	0.03	1.16	0.02	1.07	0.06
MTDH	1.01	0.01	0.99	0.03	0.90	0.04
RPS3A	1.07	0.09	1.00	0.13	0.94	0.02
KIAA0063	2.64	0.17	2.58	0.14	2.48	0.13
ARMET	2.67	0.14	2.65	0.17	2.41	0.02
GPX1	3.01	0.15	3.21	0.25	2.83	0.29
FBLN5	8.14	0.87	7.66	0.71	6.06	1.53
A_23_P123234	16.22	0.82	15.45	0.67	14.00	1.35
MARCKS	7.12	0.18	7.19	0.39	6.43	0.26

(Table 1). Using the class comparison method provided in BRB-Array Tools, we determined that there were no significantly changed genes among the first and second use of the microarray with a false discovery rate <5%, and only three genes were found to be altered in their expression between the second and third hybridization. This comparison looked at all genes across the three replicates and established that they can be used up to three times. Selected specific genes are listed in Table 2 that cross the three hybridizations, indicating that the derived ratios are comparable. Since we observed some reduction of signal after the second use, as a practical consideration, reliable results are obtained by comparison between samples at the same reuse level. This reduction in signal intensity has little effect on ratios that were statistically different as determined by BRB Array Tools, because reuse affects genes that are expressed at low abundance and normally do not show up as statistically significant alterations in gene expression.

These data showed that the RNase H stripping of oligonucleotide microarrays was possible with high reproducibility. The second and third hybridized arrays showed replication correlation coefficients as high as the first hybridized arrays.

Here, we report a technique for stripping oligonucleotide microarrays using RNase H. This method is relatively milder (stripping temperature at 37°C and pH 8.0) than alkaline-based stripping protocols. The same array can be used up to three times with a satisfactory correlation coefficient, which helps to reduce the cost of microarray studies. This technique may be applicable to other types of oligonucleotide or DNA microarrays hybridized with sense or antisense RNA.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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