Optimizing stringency for expression microarrays

James E. Korkola, Anne L.H. Estep, Sunanda Pejavar, Sandy DeVries, Ronald Jensen, and Frederic M. Waldman

BioTechniques 35:828-835 (October 2003)

While several studies have reported methods to optimize expression microarray protocols, none have dealt directly with hybridization wash stringency. We designed a series of experiments to determine the optimal stringency conditions for microarray experiments, using reproducibility and magnitudes of log_2 (test/reference) ratio values as measures of quality. Low-stringency wash conditions of cell line hybridizations led to nonspecific binding, resulting in increased intensities, decreased magnitude of ratios, and poor reproducibility. Relatively high-stringency wash conditions were found to give the best reproducibility and large magnitude ratio changes, although increasing the stringency beyond this point led to lower magnitude ratios and poorer reproducibility. The expression levels of the ERBB2 oncogene in the BT474 versus MCF7 cell lines showed that high-stringency wash conditions gave the best agreement with real-time quantitative PCR, although the magnitude of the changes by microarray was smaller than for real-time quantitative PCR. Analysis of a series of cell lines washed at the optimized stringency indicated that the rank order of relative expression levels for ERBB2 microarray clones agreed well with the rank order of ERBB2 levels, as measured by quantitative PCR. These results indicate that the optimization of stringency conditions will improve microarray reproducibility and give more representative expression values.

INTRODUCTION

The ability to survey thousands of genes in a single sample and to recognize complex patterns in related samples that predict parameters, such as gene function (1), clinical response (2), or cell cycle phases (3), has made the microarray an important discovery tool. A number of studies have reported methods for optimizing microarray protocols. These include reports on the preparation of arrays and labeling of probe (4), amplification of RNA for use in microarrays (5), and analysis of the resulting data (6). Although several reports have briefly discussed stringency (7,8), no reports have directly examined stringency effects on cDNA microarray results. The optimization of stringency conditions should improve the results from array experiments. If conditions are too stringent, then specific signal will be washed from the microarray, leading to the loss of signal and therefore lower signal-to-noise ratios. Conversely, low-stringency conditions will lead to nonspecific probe binding and masking of specific signal.

We designed an experiment to test the effects of wash stringency on microarray results. A range of wash conditions with different stringencies was tested using four breast cell lines and a reference pool hybridized against itself. High-stringency wash conditions gave the best results, with better reproducibility and ratios with larger magnitude changes than lower stringency washes. Analysis of the ERBB2 gene indicated that at the best stringency condition, there was good agreement between real-time quantitative PCR and microarray results. These results show that high-stringency washes improve expression microarray reproducibility and give more representative expression values.

MATERIALS AND METHODS

Samples

Breast cancer cell lines BT474, MCF7, T47D, and MDA231 (ATCC, Manassas, VA, USA) were obtained from the University of California San Francisco (UCSF) Cell Culture Facility (San Francisco, CA, USA). RNA was isolated from subconfluent flasks using TRIZOL® Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA quantity and quality were determined by using ultraviolet absorption at 260 nm and by running 100 ng RNA on denaturing agarose gels. The RNA was then DNase-digested using DNA-*free*™ (Ambion, Austin, TX, USA). A reference cell line RNA pool for the microarray hybridizations was prepared using TRIZOL and consisted of equal amounts of RNA from the following cell lines: MCF7 (breast adenocarcinoma); HepG2 (hepatocellular carcinoma); MOLT-4 (T cell leukemia); NTERA-2 (teratoma); SW872 (liposarcoma); WM115 (melanoma); OVCAR3 (ovarian carcinoma); RPMI8226 (mutiple myeloma); Colo205 (colon adenocarcinoma); Hs578T (breast carcinosarcoma); and HL60 (acute promyelocytic leukemia) (ATCC).

University of California San Francisco, San Francisco, CA, USA

Table 1. Wash Conditions and Resulting Stringencies

Wash No.	Buffer	Wash Temperature (°C)	Calculated T_m [a] (°C)	ΔT [b] (°C)
1	2× SSC/1%SDS	20	93	73
		42	93	51
		55	93	38
		65	93	28
2	2× SSC	20	93	73
3	0.2× SSC	20	71	51

SSC, standard saline citrate; SDS, sodium dodecyl sulfate; T_m, melting temperature; ΔT, stringency.

[a] $T_m = 81.5°C + 16.6\log[Na^+] + 0.41(\%GC) - 0.63(\%\text{ formamide}) - (600/\text{length})$.

[b] $\Delta T = T_m - T_{wash}$.

Microarray Preparation

Arrays composed of 6144 cDNA clones [or 10,368 for the TaqMan® (Applied Biosystems, Foster City, CA, USA) real-time quantitative PCR comparison experiment] from the Research Genetics 40K clone set (Research Genetics, Huntsville, AL, USA) were prepared using standard methods (3). Briefly, bacterial stock was PCR-amplified directly using primers as previously described (4). The cDNA product was purified by ethanol precipitation, redissolved in 3× standard saline citrate (SSC), and arrayed into 384-well microplates. The cDNAs were then robotically spotted onto poly-L-lysine-coated glass slides. Following printing, the arrays were post-processed using standard methods that have been previously described (http://www.microarrays.org).

Labeling and Hybridization

Ten micrograms of total RNA from each cell line were reverse-transcribed with SUPERSCRIPT™ II Reverse Transcriptase (Invitrogen) in the presence of aminoallyl-modified dUTP (Sigma, St. Louis, MO, USA) using 2 µg random hexamers (Invitrogen) and 1.25 µg oligo(dT) primers (Invitrogen). Bovine serum albumin (BSA) (1.5 µg; Ambion) and 125 µmol ddATP (Invitrogen) were also included in the reaction to increase the signal and representation, respectively, of rare transcripts (9). The cDNA products were then labeled by coupling to free Cy™3 dye and mixing with a Cy5-labeled reference (both from Amersham Biosciences, Piscataway, NJ, USA). The labeled probe was then purified using QIAquick™ PCR columns (Qiagen, Valencia, CA, USA) and mixed with 2 µg Cot-1 DNA and 10 µg tRNA in a 25 mM HEPES buffer solution, pH 7.0, with a final concentration of 3× SSC and 0.03% sodium dodecyl sulfate (SDS). The probe was heated and applied under a lifter slip (Erie Scientific, Portsmouth, NH, USA) to the slide surface. The slides in any one experiment were all from the same print run to minimize variability. The slides were incubated in a 65°C water bath overnight in a HybChamber™ (GeneMachines, San Carlos, CA, USA).

Stringency Calculations and Washes

Following overnight hybridization, the slides were subjected to three washes for 10 min each. Wash 1 was in 2× SSC/0.1% SDS and was varied to define specific stringencies [performed at 20° (room temperature), 42°, 55°, or 65°C]. Wash 2 was in 2× SSC, and wash 3 was in 0.2× SSC, both at room temperature for all experiments. The slides were kept separate for the second and third washes so that slides from each stringency level were never washed together. During wash 1, the slides were agitated several times, while washes 2 and 3 were mixed constantly with a stir bar.

The stringencies of each wash were calculated using the following formula (10) for melting temperature (T_m):

$$T_m = 81.5°C + 16.6\log[Na^+] + 0.41(\%GC) - 0.63(\%\text{ formamide}) - (600/\text{length})$$

where $[Na^+]$ is the sodium concentration (0.33 M for 2× SSC); %GC is the percentage of guanine and cytosine in the DNA (assumed to be 50% for this experiment); % formamide is the volume percentage of formamide in the solution (0 for these experiments); and length is the length of the DNA probe that is being hybridized (assumed to be 600 bp for these experiments). The stringency can be calculated by subtracting the wash temperature from the calculated melting temperature, as follows: $\Delta T = T_m - T_{wash}$. As ΔT is decreased, the stringency increases. Table 1 shows the wash conditions and calculated stringencies for each of the conditions used. The four temperatures for wash 1 represented ΔTs of 73° (20°C wash), 51° (42°C wash), 38° (55°C wash), and 28°C (65°C wash). Both the second (ΔT = 73°C) and third (ΔT = 51°C) washes were performed at room temperature (20°C). After the third wash, the slides were briefly rinsed in distilled water and then dried with compressed air.

Imaging and Analysis

Slides were imaged on a GenePix® 4000B scanner (Axon Instruments, Union City, CA, USA) using standard photomultiplier tube settings to allow for the easy comparison of intensities between slides (532 nm at 550 V; 635 nm at 700 V). The images were analyzed using GenePix Pro version 3.06 software (Axon Instruments). Background-subtracted data were centered using subarray median, followed by global loess correction as part of the Bioconductor R package (http://www.bioconductor.org). Clustering was done using Michael Eisen's Treeview/Cluster software package (http://rana.lbl.gov) (11). The clones were selected for reproducibility testing based on their presence in multiple copies on the arrays and showing changes averaged across the four stringency conditions for each cell line.

Real-Time Quantitative PCR Analysis

Real-time quantitative PCR was performed on the MCF7 and BT474 samples from the reference pool to compare to the microarray results. Briefly, 100

ng total RNA were reverse-transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase and random hexamer primers (both from Invitrogen), according to standard protocols (12). The samples were then subjected to real-time PCR analysis using an ABI PRISM® 7700 real-time thermal cycler (Applied Biosystems) at the UCSF Comprehensive Cancer Center Genome Analysis Core. The probes used were ERBB2, β-glucuronidase (β-Gus), and GAPDH.

RESULTS

Stringency Effects on Intensity

Table 2 shows the average intensity for test and reference channels, overall standard deviation of the $\log_2$(test/reference) ratios, and the number of spots included (as a measure of spot intensity, related to nonspecific probe binding for low-expressing genes) for the samples at different stringency conditions. The standard deviation is a reflection of the heterogeneity of expression levels on the array. For every sample, the overall standard deviation of the $\log_2$(test/reference) ratios was lowest at the lowest stringency, increased for the next two stringency levels, and decreased for the highest stringency level. The total number of spots included in each hybridization tended to decrease from low stringency to high stringency. The average intensities were significantly higher in the samples washed at the lowest stringency ($P < 0.01$). For the other wash conditions, the intensities did not differ from each another.

The average background levels for both Cy5 and Cy3 did not change with stringency. The average of the median background intensities for Cy5 across the five different cell lines were 204 (20°C), 181 (42°C), 203 (55°C), and 189 (65°C). For Cy3, the average background intensities were 308 (20°C), 314 (42°C), 321 (55°C), and 322 (65°C).

Reproducibility and Ratio Magnitudes

For each cell line, we selected clones that showed changes ($\log_2$ ratios greater than 1 or less than -1) and were represented multiple times on the arrays to define the reproducibility of array results. These clones were used to calculate the means and standard deviations. There were 42 clones that were present with multiple copies printed on the arrays that showed alterations in at least one cell line. There were 31 clones in BT474, 19 clones in MCF7, 25 in MDA231, and 19 in T47D that met these criteria. Table 3 shows the average standard deviations for each cell line and the reference pool at each stringency level. The average of the standard deviations of the $\log_2$(test/reference) ratios for the selected clones was lowest when the slides were washed at 20°, followed by 55°, 42°, and 65°C, respectively.

In addition to clones that were present multiple time that showed changes, we also examined all the clones that showed a $\log_2$ ratio change averaging greater than 1 or less than -1 in a cell line, regardless of how many copies were present. The ratio values for these clones were plotted for each cell line by stringency level, as shown in Figure 1. The number of clones that fit this criterion varied by cell line, with BT474 having the most clones that were above 1 or below -1, and MCF 7 having the fewest. A moving average was calculated to smooth the plot to make visualization easier (the ratio value for each clone was averaged with the two previous and two following ratio values). In each case, arrays washed at 55°C showed the largest magnitude changes for clones that had low levels of expres-

Table 2. Quality Measures of Expression Array Hybridizations

Sample	Condition (°C)	Cy3[a]	Cy5[b]	SD Test/ Reference[b]	Spots[c] (*n*)
BT474	20	2712	5036	0.436	5528
	42	1150	1186	0.650	5658
	55	1240	1251	0.701	5497
	65	1026	1540	0.600	5512
MCF7	20	3690	8097	0.226	5571
	42	754	1870	0.472	5666
	55	638	1420	0.587	5537
	65	875	1996	0.508	5535
MDA231	20	2535	4906	0.337	5591
	42	864	1380	0.520	5632
	55	1077	1343	0.600	5509
	65	989	1568	0.550	5233
T47D	20	1552	2100	0.425	5522
	42	1240	1721	0.550	5681
	55	1001	1274	0.686	5223
	65	926	1417	0.555	5378
Reference	20	2452	5079	0.187	5640
	42	1109	1749	0.247	5521
	55	810	1271	0.291	5053
	65	877	1619	0.286	5359

SD, standard deviation.
[a]Average of mean intensity (background-subtracted) for all spots included in analysis.
[b]Overall standard deviation of all corrected $\log_2$(test/reference) ratios.
[c]Total number of cDNAs included in analysis.

Table 3. Average Standard Deviations of Selected Clones at the Four Stringency Levels

	Wash No. 1 Temperatures			
Sample[a]	**20°C**	**42°C**	**55°C**	**65°C**
BT474 (n = 31)	0.278[b]	0.385	0.261	0.305
MCF7 (n = 19)	0.235	0.313	0.353	0.338
MDA231 (n = 25)	0.296	0.301	0.313	0.418
T47D (n = 19)	0.192	0.347	0.284	0.335
reference (n = 42)	0.141	0.165	0.117	0.172
Average	0.228	0.302	0.266	0.314

[a] n represents the total number of clones that showed changes and were represented multiple times on the arrays for each cell line.

[b] Values represent the average of the standard deviations for each set of replicate clones for a given cell line at the different wash temperatures.

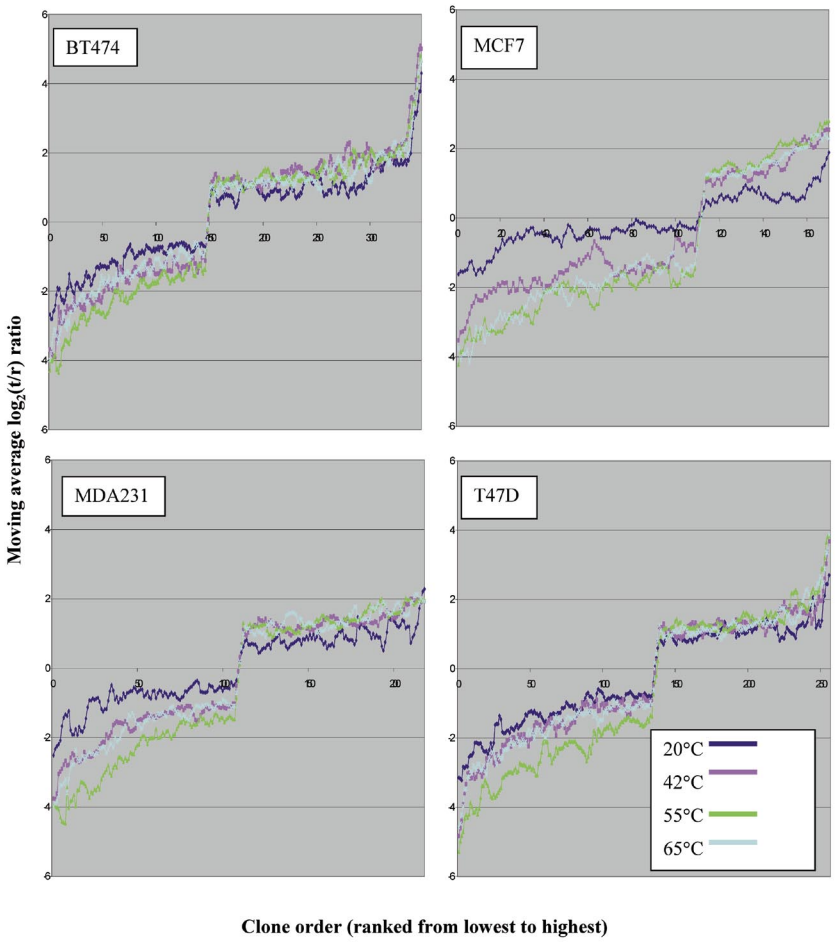

Figure 1. Moving average $\log_2$(test/reference) ratios of all clones that showed alterations for each stringency level. To smooth the curve, a moving average was calculated by averaging the ratio for a given clone with the two previous and two following ratios. The x-axis represents the clones ordered from the lowest average expression to highest average expression.

sion. For clones showing high levels of expression, there did not appear to be any differences between washes at 42°, 55°, or 65°C. For both low- and high-expressing clones, the arrays washed at 20°C had the lowest magnitude ratios.

Stringency Effects on Clustering

The four sets of cell line and reference samples were clustered using Eisen's Treeview/Cluster software package. The data were centered to a $\log_2$ ratio of zero. No further normalizations were performed. Figure 2 shows the clustering of samples. Note that for MCF7, the sample washed at 20°C did not cluster with the MCF7 samples washed at higher stringencies. Instead, this sample was higher up the cluster dendrogram, clustering with the MCF7, BT474, and MDA231 samples. The BT474 and T47D samples did not show any significant effect of stringency on clustering, but the sample washed at 20°C for MDA231 was the most weakly clustering sample.

ERBB2 Array Data versus Real-Time Quantitative PCR

The BT474 breast cancer cell line is known to overexpress the *ERBB2* oncogene, while the MCF7 breast cancer cell line shows normal levels of expression. TaqMan measures *ERBB2* expression 125× higher in BT474 than in MCF7. The levels of *ERBB2* in BT474 compared to MCF7 from microarrays were calculated for each stringency level. The 20°C wash showed the lowest ratio (17.9:1), followed by the 42°C wash (22.6:1), 65°C wash (23.0:1), and 55°C wash (32.2:1).

Washes at 55°C were determined to be optimal due to the best reproducibility and largest magnitude ratios. A series of hybridizations were done to compare hybridization results at this stringency level to real-time quantitative PCR data. Each of the 11 cell lines in the reference pool plus BT474 was hybridized versus the reference pool to arrays, which consisted of 10,368 cDNA clones. The normalized relative levels of expression of *ERBB2* for two sequence-verified clones of this gene were compared to relative real-time quantitative PCR levels. Figure 3 shows the level of expression for the *ERBB2* gene (relative to HL60, which was defined as a level of 1 for both arrays and real-time quantitative PCR) across 11 separate cell lines. The data shown are the expression levels for the two separate cDNA clones and real-time quantitative PCR (normalized to either β-*Gus* or *GAPDH*). The cell lines were ordered from lowest to highest expression, and the order as measured by the expression array was the same for both cDNA clones. The order for real-time quantitative PCR quantitation differed depending on the control gene used for normalization, as can be seen in Figure 3. The order for the cDNA clones agreed well with the real-time quantitative PCR levels relative to either control gene, although the magnitude of expression differences was less by microarray analysis.

DISCUSSION

The wash stringency can have a profound effect on expression array hybridization results. Stringency levels need to be high enough to prevent nonspecific binding, but not so high that specific signal is removed by the wash. We designed a study to examine the effects of wash stringencies on microarray results, using the magnitude of changes, reproducibility, and clustering as measures of the quality of hybridization results.

Stringency can be altered by changing the salt concentration, adding formamide, or altering temperature. We chose to alter one of these parameters, keeping the others constant to more easily interpret the results. Thus, we varied the temperature as a means of testing different stringency levels during microarray washes.

The first measure of quality was the standard deviation of the log(test/reference) ratios of the spotted cDNA clones. If the stringency is too low, then nonspecific binding will occur, leading to a lower overall average standard deviation of all the log(test/reference) ratios for an experiment and the inclusion of more spots. If stringency is too high, then specific signal will be washed off, which will decrease the signal intensity above background. This would likely result in poorer reproducibility, a smaller number of spots passing quality controls, and may decrease the magnitude of ratios. The ideal stringency should give a large standard deviation in a cell line versus reference hybridization (the largest standard deviation will give the largest spread of ratio values) with good reproducibility and the inclusion of the most spots. We found that the washes at 55°C (ΔT = 38°C) gave the highest standard deviations in all the cell lines, indicating that it may be the best of the conditions tested. Washes at room temperature (20°C; ΔT = 73°C) showed evidence of nonspecific binding, with standard deviations that were comparable to reference versus reference hybridizations for the MCF7 and T47D hybridizations. The two remaining stringency levels showed consistently lower standard deviations than the 55°C wash level, indicating that there may have been loss of specific signal and increase in noise for the highest stringency wash or nonspecific binding for the lower stringency wash. The interpretation of these results is that there is some nonspecific binding with a 42°C first wash (ΔT = 51°C) and some removal of specific binding with a 65°C first wash (ΔT = 28°C).

The average Cy3 and Cy5 intensities varied by stringency level. The slides with the lowest stringency showed the

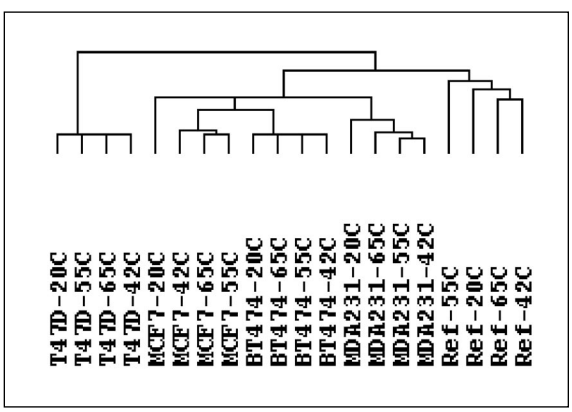

Figure 2. Hierarchical clustering of samples. Unsupervised clustering was performed for four cell lines and the reference sample washed under four different conditions, as indicated by the different temperatures. Note that the 20°C sample for MCF7 does not cluster with the higher stringency MCF7 samples.

greatest intensity values, indicating nonspecific binding. There were no differences between the intensities for the slides washed at the other stringency levels. This was somewhat surprising, given that the 42°C wash exhibited characteristics of nonspecific binding, while the 65°C wash showed some signs of the removal of specific binding.

The background intensity did not change with increasing stringency but remained constant across all stringency levels. This result is not surprising because the same concentration of SDS was present in wash 1 for all stringency levels. SDS is a detergent that should prevent probes from sticking to the surface of the slide, but it has little effect on nucleic acid hybridization.

We expected that when three serial washes are used (as in this experiment), the most stringent condition would determine overall stringency. This was not the case in these experiments. As can be seen in Table 1, samples washed initially at either 20° or 42°C had a minimum ΔT of 51° (third wash for 20°; first and third washes for 42°C). However, the results from the experiments clearly indicate that the two wash conditions were not identical. This may reflect the time spent in the highest stringency wash (two 10-min washes for the samples initially washed at 42°C, compared to one 10-min wash for the sample initially washed at 20°C).

To determine the stringency effects on the reproducibility and magnitude of the changes of the genes, a series of specific genes were chosen for quantitative analysis. These genes showed alterations in expression ($\log_2$ test/reference ratio averaging greater than 1 or less than -1 across the stringency levels) in the specific cell line and were represented multiple times on the arrays. The standard deviation for each set of replicate genes was calculated, and an average standard deviation of all the clones was determined for each of the cell lines. Two of the four cell lines (BT474 and T47D) had the lowest standard deviations among replicates when washed at 55°C. The remaining two cell lines (MCF7 and MDA231) had the lowest standard deviation among replicates when washed at 42°C. The clones had the lowest standard deviation in the reference when the washes were performed at 55°C. These results indicate that good reproducibility could be obtained when washes were performed at either 42° or 55°, but that 55°C washes gave slightly

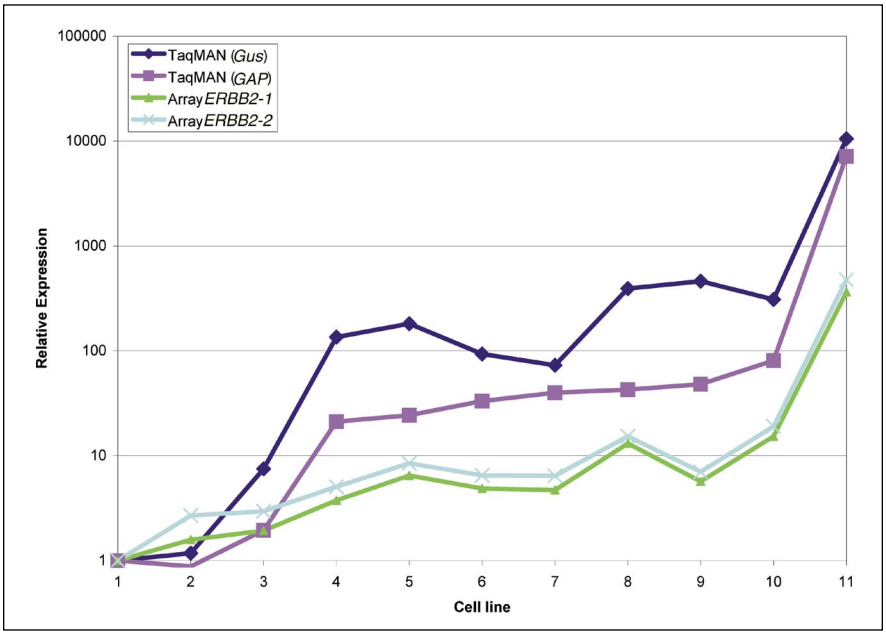

Figure 3. Relative expression levels for the *ERBB2* gene measured by microarray or real-time quantitative PCR analysis. All samples were washed at the 55°C stringency condition. Real-time quantitative PCR was normalized relative to either *GAPDH* or β-glucuronidase (β-*Gus*). Expression for all samples is relative to HL-60 (set to 1). The cell lines used are as follows: 1, HL60; 2, RPMI 8226; 3, MOLT-4; 4, Hs578t; 5, NTERA-2; 6, MCF7; 7, SW872; 8, HepG2; 9, OVCAR3; 10, WM115; 11, BT474.

better reproducibility.

When the magnitude of the changes was examined for all the genes, the magnitude of the change was greatest when the washes were performed at 55°C for most clones (Figure 1). This was particularly true for clones that showed low levels of expression relative to the reference. For clones that showed high levels of expression relative to the reference, the result was not as clear. While 55°C washes clearly showed a larger magnitude in the ratio than washes at 20°C, the differences between 42°, 55°, and 65°C washes were minor. The differences between the high-expressing clones varied more than the low-expressing genes, with the sample with the largest magnitude ratio varying, depending on the clone as opposed to the wash stringency. There were also significant differences depending on which cell line was being examined, with MCF7 showing higher magnitude changes at 55°C than at the other high-stringency conditions for the high-expression clones. The other cell lines did not show such differences. The variability observed is likely due to the complex nature of the probe being hybridized. The stringency depends on factors such as the %GC content and the length of the probes. For each 1.0°–1.5°C drop in melting temperature, there is a 1% increase in mismatch allowed (10). Thus, for a probe-target hybrid that is 600 bp in length, with a %GC content of 50%, the 55°C wash condition would allow for an approximate 25% mismatch between the probe and target, while the 42°C wash would allow for a 34% mismatch. Because the length and %GC are not fixed values for microarrays, the percentage of mismatches will vary for each probe-target hybrid. On average, 55°C washes give the best reproducibility and largest magnitude changes for genes that have low expression and the best reproducibility for high-expressing genes with comparable magnitude changes. The reason why the low-expressing genes show a consistent difference while high-expressing genes do not is unclear but may be due to different binding kinetics based on the Cy5 label compared to Cy3 label.

The clustering of the samples was done using Eisen's Treeview/Cluster software. Normalization was not done on these samples because they were not washed under the same conditions. The normalization of each sample is usually performed to ensure that the standard deviations of each sample are equivalent to correct for factors such as labeling efficiency and hybridization kinetics. In this case, normalization was not appropriate because stringency was expected to have a direct effect on the overall standard deviation, and this was one of the variables being tested. Furthermore, reference versus reference samples was included in the clustering, and normalization would be inappropri-

ate for such samples. The clustering of non-normalized samples shows that the 20°C washed sample for MCF7 does not cluster with the higher stringency MCF7 samples. For MDA231, the 20°C wash was also the most weakly clustering sample, as shown by the distance on the clustering dendrogram.

Real-time quantitative PCR analysis was performed on the breast cell lines BT474 and MCF7 for the *ERBB2* oncogene. This analysis showed that BT474 expressed approximately 125× as much *ERBB2* as MCF7. A comparison of the array-based BT474:MCF7 levels for the *ERBB2* gene showed that the 55°C wash came closest to this level. Real-time quantitative PCR analysis of the expression levels in a number of other cell lines for the *ERBB2* gene was performed for comparison to the relative levels, as determined by microarray experiments at the optimized 55°C wash stringency. Ranking of the cell lines by *ERBB2* levels showed that the order was similar for real-time quantitative PCR compared to two separate microarray clones. The rank order for the two clones was identical, whereas the real-time quantitative PCR rank order varied depending on which housekeeping gene (β-*Gus* or *GAPDH*) was used for normalization. The levels of expression were less for microarrays than for real-time quantitative PCR, which is likely due to hybridization effects such as background, spot saturation, steric effects, and dye effects.

These results are limited to the cDNA platform and two-color hybridizations. Alternative approaches use oligonucleotide arrays and one- or two-color analyses. Oligonucleotides have hybridization characteristics that are different from cDNAs because oligonucleotides have high specificity (unique sequence) but short lengths, which affects the hybridization and melting of double-stranded DNA. The results of this study are not directly applicable to oligonucleotide arrays, but stringency conditions with these arrays should also be optimized and validated.

We show the importance of optimizing stringency in microarray experiments. The stringency must be a balance between intensity and specificity, so that the signal is maximized while minimizing nonspecific probe binding. The results presented here indicate that high-stringency washes in microarray experiments are preferable to low-stringency washes. The best condition found in these experiments was higher than is typically used in cDNA microarray experiments, which raises the possibility that experiments performed at lower stringency may have missed some differentially expressed genes. Changing current protocols to optimize stringency would likely improve reproducibility. High-stringency washes result in higher magnitude ratios, better reproducibility in expression microarray experiments, and expression values that are more representative of true cellular mRNA quantities.

ACKNOWLEDGMENTS

The authors wish to thank Dr. J. DeRisi for use of his arrayer robot, Jane Fridlyand for help with Bioconductor R, and Dr. R. Kramer for use of the WM115 melanoma cell line. This work was supported by Komen Foundation grant no. BCTR00-000378 (F.M.W.).

REFERENCES

1. **Hughes, T.R., M.J. Marton, A.R. Jones, C.J. Roberts, R. Stoughton, C.D. Armour, H.A. Bennett, E. Coffey, et al.** 2000. Functional discovery via a compendium of expression profiles. Cell *102*:109-126.
2. **van 't Veer, L.J., H. Dai, M.J. van de Vijver, Y.D. He, A.A. Hart, M. Mao, H.L. Peterse, K. van der Kooy, et al.** 2002. Gene expression profiling predicts clinical outcome of breast cancer. Nature *415*:530-536.
3. **Spellman, P.T., G. Sherlock, M.Q. Zhang, V.R. Iyer, K. Anders, M.B. Eisen, P.O. Brown, D. Botstein, et al.** 1998. Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. Mol. Biol. Cell *12*:3273-3297.
4. **Hegde, P., R. Qi, K. Abernathy, C. Gay, S. Dharap, R. Gaspard, J.E. Hughes, E. Snesrud, et al.** 2000. A concise guide to cDNA microarray analysis. BioTechniques *29*:548-562.
5. **Baugh, L.R., A.A. Hill, E.L. Brown, and C.P. Hunter.** 2001. Quantitative analysis of mRNA amplification by in vitro transcription. Nucleic Acids Res. *29*:e29.
6. **Brazma, A. and J. Vilo.** 2000. Gene expression data analysis. FEBS Lett. *480*:17-24.
7. **Relogio, A., C. Schwager, A. Richter, W. Ansorge, and J. Valcarcel.** 2002. Optimization of oligonucleotide-based DNA microarrays. Nucleic Acids Res. *30*:e51.
8. **Hughes, T.R., M. Mao, A.R. Jones, J. Burchard, M.J. Marton, K.W. Shannon, S.M. Lefkowitz, M. Ziman, et al.** 2001. Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. Nat. Biotechnol.*19*:342-347.
9. **Decraene, C., I. Reguigne-Arnould, C. Auffray, and G. Pietu.** 1999. Reverse transcription in the presence of dideoxynucleotides to increase the sensitivity of expression monitoring with cDNA arrays. BioTechniques *27*: 962-966.
10. **Maniatis, T., E.F. Fritsch, and J. Sambrook.** 1989. Molecular Cloning: A Laboratory Manual. CSH Laboratory Press, Cold Spring Harbor, NY.
11. **Eisen, M.B., P.T. Spellman, P.O. Brown, and D. Botstein.** 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA *95*:14863-14868.
12. **Ginzinger, D.G.** 2002. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp. Hematol. *30*:503-551.

Received 27 May 2003; accepted 23 July 2003.

Address correspondence to:

Frederic M. Waldman
UCSF Comprehensive Cancer Center
2340 Sutter Street, Rm. 436
San Francisco, CA 94143-0808, USA
e-mail: waldman@cc.ucsf.edu