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ORIGINAL ARTICLE

Prediction of qualitative outcome of oligonucleotide microarray hybridization by measurement of RNA integrity using the 2100 Bioanalyzer™ capillary electrophoresis system

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Abstract RNA quality is critical to achieve valid results in microarray experiments and to save resources. The RNA integrity number (RIN) can be measured with minimal sample consumption by microfluidics-based capillary electrophoresis. To determine whether RIN can predict the qualitative outcome of microarray hybridization, we measured RIN in total RNA samples from 484 different experiments by the 2100 Bioanalyzer system and correlated with the percentage of present calls (%pc) of downstream oligonucleotide microarrays. The correlation coefficient for RNA and %pc in all 408 samples for which the bioanalyzer algorithm was able to produce an RIN was 0.475 ($p<0.05$), ranging from 0.039 to 0.673 for different tissue- and assay-type subgroups. Multivariate analysis found RIN to be the best predictor of microarray quality as assessed by %pc, outperforming the 28S to 18S ratio. For a %pc threshold of 25% and 35%, we determined optimal cut points for RIN at 7.15 and 8.05, respectively. Using the suggested cut points, RIN can support the final decision whether a certain RNA sample is appropriate for successful microarray hybridization.

Keywords RNA quality · RIN · Capillary electrophoresis · Bioanalyzer · Oligonucleotide microarray · Present calls

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Introduction

RNA oligonucleotide microarray platforms are increasingly used to create gene expression profiles of tissues involved in various medical conditions, particularly in hematological and oncological diseases. The derived data help to understand the biology, facilitate diagnosis, or predict treatment response and prognosis of the disease studied.

The quality of RNA recovery and sample processing is of utmost importance to achieve valid results and to save precious resources, particularly when limited amounts of RNA are available. RNA preparations can be contaminated by DNA or protein, and they are constantly compromised by degradation. While moderate RNA degradation may still yield acceptable microarray results, extensively degraded samples should be excluded from analysis [1]. Conventional methods to assure RNA integrity include gel electrophoresis under denaturing conditions with determination of the 28S and 18S ribosomal RNA band ratio and UV spectrometry determining the ratio of absorbance at 260 and 280 nm (optimal ratio 1.8–2.1). Major drawbacks of these methods are either large quantities of RNA required for analysis, dependence on electrophoresis conditions, or the inability to detect DNA contamination.

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The Agilent 2100 Bioanalyzer™ (Agilent Technologies, Santa Clara, CA, USA) is a microfluidics-based platform based on capillary electrophoresis that can be used to quantify as well as to quickly and reliably assess quality of RNA samples with minimal sample consumption [2]. In addition to the calculation of a 28S to 18S rRNA ratio, the system software includes an algorithm to calculate an RNA integrity number (RIN), a value between 1 and 10 in one-decimal steps. Using RIN, sample integrity is determined by the entire electrophoretic trace of the RNA sample instead of the 28S to 18S rRNA ratio alone. RIN can be used as a standardized measure to correlate with results from subsequent microarray experiments and determine thresholds for meaningful results [3].

Quality of oligonucleotide microarray results can be estimated by several metrics provided by an Affymetrix software report file:

1. *Percentage of present calls* (%pc), an array level summary of the results of a statistical function designed to predict the presence or absence of each gene transcript [4]. It can be used as a quality metric that is sensitive to any error source from RNA sampling to scanning and data extraction and is therefore cumulatively influenced by all stages in the microarray process. Furthermore, different array classes, brightness, background measures, and detection algorithms greatly influence this quality metric by up to 40% [5]. Different values have reportedly been used as a threshold for poor-quality assays. Finkelstein et al. used 25% as a threshold for outliers, a value which is also recommended by the Tumor Analysis Best Practices Working Group [6], whereas Weis et al. [7] found a value below 35% to correlate with poor-quality assays in their experiments.
2. The *average background* is calculated from the 2% of probes with the weakest signal. It is an estimate of general nonspecific binding based on low-intensity features across an array.
3. *Bio B* is a probe set designed to measure prelabeled bacterial nucleotides. It is the signal from internal prelabeled standards and measures the efficacy of hybridization, washing, and scanning. Bio B is free of RNA, amplification, and labeling effects.
4. The 3′ to 5′ ratio of a housekeeping gene (e.g., glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or beta-actin) is a ratio of probe sets designed to detect the 3′ and 5′ regions of the messenger RNA transcript of a certain housekeeping gene and is reputed to detect RNA degradation. This ratio is thought to indicate RNA quality as well as the bias inherent in the Affymetrix labeling assay and should be below a value of 3 [8].
5. The *scale factor* is a global normalization constant based on the trimmed mean of probe set signals or average differences and is inversely related to array brightness.

Except for Bio B, all quality metrics are influenced by cumulative errors.

Most quality control measures are not entirely independent of each other. Percentage of present calls and 3′ to 5′ ratio most significantly correlate with RNA sample quality.

The aim of the present study was to see if RNA integrity measured by RIN can predict microarray quality. Using a large database of oligonucleotide microarray experiments including samples with different tissue origins as well as cell cultures, RIN was retrospectively correlated with the percentage of present calls in downstream microarrays. Other factors potentially correlating with the percentage of present calls like 28S to 18S ratio, RNA concentration, assay, and tissue type were included in a multivariate linear regression model. Cut point analysis for RIN predicting %pc below certain thresholds was performed to support decisions in future experiments of whether a sample is suitable for successful microarray hybridization.

Material and methods

Samples

Four-hundred and eighty-four RNA preparations were electrophoretically analyzed using the Agilent 2100 Bioanalyzer™ system. In 408 of these samples (84%), an RIN could be calculated using the Agilent 2100 Bioanalyzer Expert Software™; in 76 samples, the algorithm failed to produce an RIN. This failure to produce a valid RIN was mostly due to a missing or displaced lower marker, extremely small amounts of RNA (<10 ng/ml), or peak shifts on the time axis despite optically immaculate electropherograms.

The samples originated from various experiments performed in our microarray laboratory from 2002 to 2005. RNA samples were prepared from cell lines, human mononuclear bone marrow cells, human CD34-selected cells, murine hematopoietic cells, and mouse tissue (Table 1). RNA was extracted by standardized protocols using either TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) or the RNeasy™ kit (Qiagen, Valencia, CA, USA) according to the manufacturers' guidelines. RNA concentration was measured with a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Table 1 Sample origin and processing

Sample type	*N* (%)	*N* with valid RIN (%)	
		Nanogram	Standard
Total	484 (100)	408 (100)	
		233 (57)	175 (43)
Cell line	195 (40)	153 (38)	
		42 (10)	111 (27)
Human bone marrow	129 (27)	114 (28)	
		94 (23)	20 (5)
Human CD34 selected	118 (24)	104 (26)	
		97 (24)	7 (2)
Mouse tissue	26 (5)	24 (6)	
		0	24 (6)
Mouse hematopoietic cells	16 (3)	13 (3)	
		0	13 (3)

Total numbers of RNA samples listed for different tissue origins and processing by standard assay or nanogram-scale assay

RIN RNA integrity number

Oligonucleotide microarrays

All RNA samples regardless of bioanalyzer output were further processed and hybridized with microarrays specific for the analyzed type of RNA (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's guidelines. Due to the limited RNA content in certain sample preparations (e.g., CD34-selected cells), a double in vitro transcription technique (nanogram-scale assay) was used in more than half of the experiments (n=233, see Table 1). To assay 50-ng total RNA, the standard Affymetrix target amplification protocol was modified by using the first-round complementary RNA (cRNA) product to generate double-stranded complementary DNA that was then used for a second round of in vitro transcription for synthesis of biotinylated cRNA [9].

Most experiments using human tissue (n=428) were performed with the HG-U133A array (Affymetrix) and a smaller amount (n=7) with the Hu6800 array (Affymetrix), and experiments with mouse tissue (n=49) were performed with the MG_U74Av2 mouse array (Affymetrix).

After hybridization, the microarray was washed and stained using an Affymetrix fluidics station and was scanned with an argon-ion confocal laser with 488-nm emission and detection at 570 nm. Fluorescence intensity was normalized to the average fluorescence for the entire microarray.

GeneChip image analysis was performed using the Microarray Analysis Suites 4.0.6 and 5.0 (Affymetrix) including the array quality assessed by the percentage of present calls.

Statistics

Correlations between RIN and %pc were calculated for all samples and separately for distinct subgroups: assay type (nanogram and standard assay), microarray type (HG-U133A, Hu6800 and MG_U74Av2), and tissue origin (cell culture, human mononuclear bone marrow cells, human CD34-selected cells, murine hematopoietic cells, and mouse tissue). Tissue origin was clustered into cell line samples and samples of other origin for multivariate analysis.

For all nonparametric correlations, Spearman's rank correlation coefficient was calculated. All given p values correspond to two-sided t tests. p values <0.05 were considered significant. All factors significantly correlating with %pc in bivariate analysis were included in a forward and backward stepwise linear regression model.

Cut points for RIN were determined by receiver operating characteristic (ROC) curves at a prespecified sensitivity of 0.8 with specificity determined as 1-false-positive rate on the horizontal axis at the curve intersection. Diagnostic accuracy was determined by measurement of the "area under the curve." It is used as a measure to indicate how well the statistical test separates poor-RNA-quality samples from good-RNA-quality samples, with an area of 0.9 to 1 representing an excellent test and an area of 0.8 to 0.9 representing a good test.

Commercially available statistical software was used (SPSS for Windows, release 15.0).

Results

Calculation and distribution of RIN

A valid RIN could be calculated in 408 samples. In 76 samples, the algorithm was not able to produce an RIN. Distribution of missing RINs to different tissues reflects their proportion within the whole sample set, but missing RINs are overrepresented in the standard assay group (61%).

Mean RIN for all experiments is 8.1 (range, 1.1–10.0). For nanogram-scale assays, the mean RIN is 7.1 (range, 1.1–10) and for standard assays 9.6 (range, 2.7–10.0), $p<0.001$. Mean RIN for cell line samples is 9.5 (range, 5.7–10.0) and for samples of other origin 7.3 (range, 1.1–10.0), $p<0.001$.

Distribution of %pc

Mean percentage of present calls is 40% (range, 5–57%). Likewise, mean %pc for samples with missing RIN is 40% (range, 8–52%). For nanogram-scale assays, the mean %pc

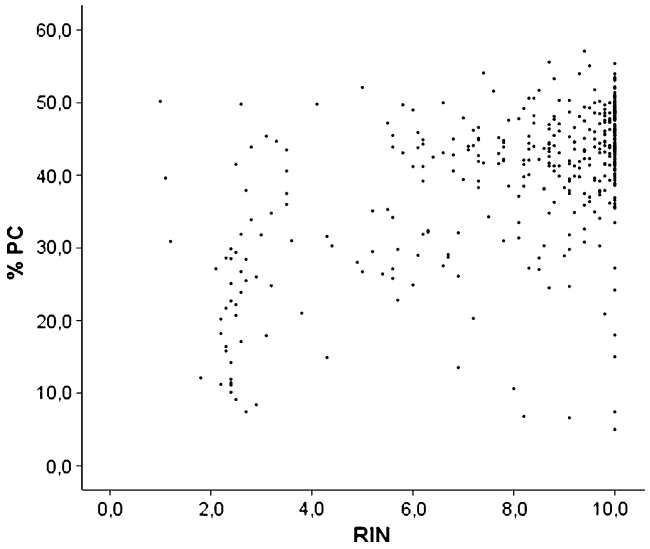

Fig. 1 Scatterplot diagram of all samples (n=408) showing correlation between RIN (*x-axis*) and %pc (*y-axis*). Overall Spearman's rank correlation coefficient is 0.475 ($p<1\times10^{-6}$). Note: *RIN*, RNA integrity number; *%pc*, percentage of present calls

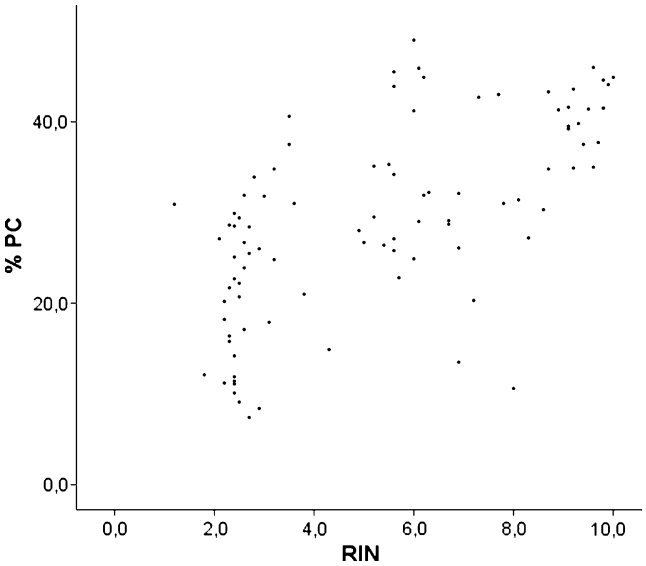

Fig. 2 Scatterplot diagram including only quality data from human mononuclear bone marrow cells processed by nanogram-scale assay (n=94) showing correlation between RIN (*x-axis*) and %pc (*y-axis*). Overall Spearman's rank correlation coefficient is 0.673 ($<1\times10^{-6}$). Note: *RIN*, RNA integrity number; *%pc*, percentage of present calls

is 37% (range, 5–55%) and for standard assays 43% (range, 7–57%), $p<0.001$. Mean %pc for cell line samples is 44% (range, 21–54%) and for samples of other origin 38% (range, 5–57%), $p<0.001$.

Correlation between RIN and %pc

For the entire sample set, a statistically significant correlation coefficient of 0.475 ($p<1\times10^{-6}$) was calculated (Fig. 1). Different coefficients were calculated for sample subgroups (Table 2). The highest correlations between RIN and %pc were found in the nanogram-scale assay group, whereas samples processed with standard assays showed no significant correlation at all. Human bone marrow samples yielded the highest correlation coefficients (Fig. 2), whereas only marginal correlations were seen in cell line preparations or CD34-selected cells. Calculations for murine samples are limited by very small numbers and have only been stated for completeness.

Correlation between 28S to 18S ratio and %pc

Data on 28S to 18S ratio were available for 453 samples. In 379 samples, both 28S to 18S ratio and RIN were available. Correlation coefficient for 28S to 18S ratio and RIN is 0.544. Correlation coefficient for 28S to 18S and %pc is 0.258 ($p<0.001$).

Table 2 Correlation between RIN and %pc

Group	Number	Mean RIN	Mean %pc	r	p
All samples	408	8.1	40.0	0.475	**$<1\times10^{-6}$**
Nanogram-scale assay	233	7.1	37.1	0.541	**$<1\times10^{-6}$**
Standard assay	175	9.5	43.7	0.069	0.363
Cell lines	153	9.5	43.7	0.283	**4×10^{-4}**
Nanogram-scale assay	42	8.7	40.8	0.291	0.061
Standard assay	111	9.7	44.8	0.075	0.436
All other tissues	255	7.3	37.7	0.578	**$<1\times10^{-6}$**
Human bone marrow	114	5.9	30.2	0.606	**$<1\times10^{-6}$**
Nanogram-scale assay	94	5.3	29.3	0.673	**$<1\times10^{-6}$**
Standard assay	20	8.8	34.7	0.039	0.87
Human CD34 selected cells	104	8.1	43.6	0.232	0.018
Mouse tissue	24	9.0	45.5	−0.158	0.462
Murine hematopoietic cells	13	9.4	42.7	0.085	0.781

Mean RIN and %pc values, Spearman's rank correlation coefficients (r), and corresponding p values (significant values in bold script) are given for all samples and subgroups defined by sample origins and assay type

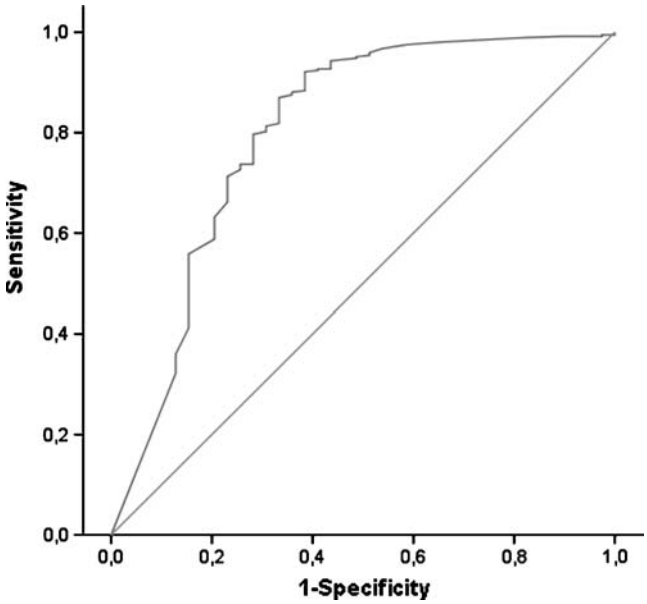

Fig. 3 Receiver operating curve plotting sensitivity (*y-axis*) and 1− specificity (*x-axis*) for a cutoff percentage of present calls of 0.25. A RIN of less or equal 7.15 predicted a %pc below 25% with the prespecified sensitivity of 0.8, a specificity of 0.7, and a diagnostic accuracy of 0.8

Influence of RNA quantity on RIN or %pc

The mean RNA concentration of all samples is 1,229 ng/μl (SD 1,804). Mean RNA concentration for nanogram-scale assays is 445 ng/μl (SD 857) and for standard assays 2,147 ng/μl (SD 2,158), $p<0.001$.

For all samples, the correlation coefficient of RNA concentration with RIN is 0.503 ($p<0.001$), and the correlation of RNA concentration with %pc is 0.316 ($p<0.001$).

The inclusion of RNA concentration as control variable in a partial correlation analysis with RIN and %pc for all samples yielded a coefficient of 0.54 indicating an even better prediction with the consideration of RNA concentration.

Linear regression model

Several factors which significantly correlated with %pc in bivariate analysis including RNA concentration, 28S to 18S ratio, tissue type (cell line or mixed tissue), assay type (standard or nanogram-scale assay), and RIN were included into a multiple linear regression model with forward and backward selection.

28S to 18S ratio and RIN remained the only predictive factors with significant prediction of %pc. Standardized coefficient (beta) in stepwise analysis was larger for RIN (0.378, $p=9\times10^{-13}$) than for 28S to 18S ratio (0.141, $p=0.006$).

Poor-quality microarrays (%pc<25% and %pc<35%)

Of 44 experiments with a present call metric (%pc) below 25%, RIN could be calculated in 39 (10% of all samples). Median RIN was 2.9 (range, 1.8–10).

Of 109 experiments with a %pc below 35%, RIN could be calculated in 93 (23% of all samples). Median RIN was 5.6 (range, 1.2–10).

In ROC analysis including all 408 valid samples, a RIN of less or equal 7.15 predicted a %pc below 25% with the prespecified sensitivity of 0.8, a sensitivity of 0.7, and a diagnostic accuracy of 0.8 (Fig. 3). A RIN of less or equal 8.05 predicted a %pc below 35% with the prespecified sensitivity of 0.8, a sensitivity of 0.73, and a diagnostic accuracy of 0.84 (Fig. 4).

Discussion

The presented systematic analysis of RNA quality and corresponding microarray quality metrics demonstrates a linear correlation between RNA integrity and the percentage of present calls, an important quality metric in microarray experiments using an Affymetrix platform with "perfect match" and "mismatch" hybridization probes. With an overall coefficient of 0.475, the observed correlation seems only moderate; however, in multivariate analysis, RIN was the most powerful predictor of microarray quality, particularly in comparison with the 28S to 18S ratio, a

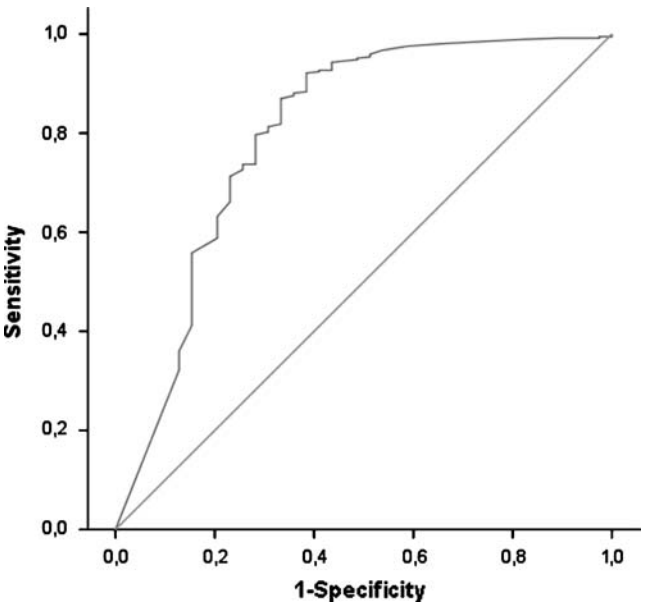

Fig. 4 Receiver operating curve plotting sensitivity (*y-axis*) and 1− specificity (*x-axis*) for a cutoff percentage of present calls of 0.35. A RIN of less or equal 8.05 predicted a %pc below 35% with the prespecified sensitivity of 0.8, a specificity of 0.73, and a diagnostic accuracy of 0.84

commonly used RNA quality metric. This is in line with a study by Copois et al. [10] who compared different methods of RNA assessment—degradometer software [11], 28S to 18S ratio, and an in-house quality scale—with array quality assessed by determination of the 3′ to 5′ ratio of GAPDH and a clustering analysis of full array expression ("dispersion tree"). A similar conclusion was drawn from a small study comparing RNA quality of 24 frozen breast cancer samples assessed by RIN, visual inspection of the capillary electrophoretic trace, and the 28S to 18S ratio [12]. Jahn et al. assessed bacterial RNA quality by RIN and found it to be critical for obtaining meaningful gene expression data. In their study, RIN values below 7.0 resulted in high variation and loss of statistical significance when gene expression was analyzed by quantitative real-time polymerase chain reaction [13].

Despite the large number of samples included in our model, analysis was confounded to a certain degree by a large proportion of cell line experiments yielding large RNA quantities with a high degree of purity. In fact, for the 153 cell line experiments with valid RIN, mean RNA quantity was 2,129 ng/μl, and mean RIN was 9.5 (54% of samples with RIN 10) compared with a mean RNA concentration of 1,214 ng/μl and mean RIN of 8.1 (30% of samples with RIN 10) in the whole sample set. Likewise, cell line experiments yielded a higher %pc (mean 43.7) compared with samples of other origin (mean 37.7).

Moreover, the different assay types are difficult to compare due to larger amounts of RNA used in standard assays compared with nanogram-scale assays. It has previously been shown that in small samples the amount of RNA entered into the experiment correlates with the percentage of present calls and other quality metrics [14]. This was reflected by a higher RIN and a greater %pc in samples processed with standard assays. Another parameter potentially influencing microarray quality is the cRNA yield further down the line of sample processing. However, in their extensive analysis of interlaboratory reproducibility of microarray experiments, Kohlmann et al. found no obvious correlation between the cRNA yield and microarray quality, concluding that multiple variables would have to be factored into a conclusion on whether a sample is suitable for microarray hybridization [15].

Due to a more even distribution of values on the RIN scale, we found samples derived from heterogeneous tissue with a higher degree of contamination to yield higher correlation coefficients compared with samples derived from cell lines. For example, considering only the subgroup of samples derived from human mononuclear bone marrow cells and analyzed with the nanogram-scale assay, we found a good correlation coefficient of 0.673. This indicates that the use of RIN to sort out poor-quality samples may be more valuable in those samples of heterogeneous origin with a greater potential for contamination.

Although RNA quantity, tissue origin, and assay type may interact with RIN and %pc, they are not independent predictors of microarray quality as seen in the linear regression model. RNA quantity has been shown to further increase the correlation of RIN and %pc in partial correlation analysis and should therefore rather be considered as an additional factor in quality prediction, however, of marginal importance.

Despite a substantial correlation, the dispersion of coordinates on the correlation curve seems rather wide. This is certainly due to a large potential for errors occurring at various stages in the experiment from RNA level to microarray data analysis. RNA contamination and degradation can occur at any of the steps following electrophoretic analysis; other reactions like biotinylation or fragmentation may confound analytic quality and last but not least array hybridization, scanning, and microarray manufacture are potential error sources for impaired array quality reflected by the percentage of present calls [16]. Thus, the assessment of sample RNA quality merely provides a snapshot at the beginning of the process, whereas the percentage of present calls is a metric of array quality incorporating the whole procedure of gene expression analysis.

Therefore, our aim was not to provide an exact numeric prediction of the percentage of present calls, which is hardly needed in microarray analysis. The larger benefit of quality prediction by RIN is the determination of a cut point. Samples with RIN values underneath that cut point are not expected to yield meaningful gene array results and, in practice, are not to be further processed. This helps to save valuable resources and improve the overall validity of results. In the literature, microarrays with a percentage of present calls below 25% or 35% are usually regarded as poor-quality arrays. Using both measures as a threshold, we found optimal cut points for RIN underneath 7.15 and 8.05, respectively. With an acceptable accuracy of 0.8 and 0.84, 11% and 30% of samples, respectively, would be left below the cut point and should be exempted from further analysis.

We have estimated that, depending on the number of samples processed on one bioanalyzer chip, RIN determination by capillary electrophoresis would be cost-effective even if only 0.3% to 4% of samples were sorted out before hybridization onto microarrays.

To conclude, we propose that RIN may be routinely used for quality prediction in microarray experiments on an Affymetrix platform utilizing "perfect match" and "mismatch" hybridization probes. The correlation with the percentage of present calls is superior to that seen with the 28S to 18S ratio. Depending on the threshold for %pc, samples with an RIN below 7.15 or 8.05 may be reliably excluded from further microarray hybridization.

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