

Use of Standard Reference Material 2242 (Relative Intensity Correction Standard for Raman Spectroscopy) for microarray scanner qualification

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BioTechniques 45:143-152 (August 2008)
doi 10.2144/000112818

As a critical component of any microarray experiment, scanner performance has the potential to contribute variability and bias, the magnitude of which is usually not quantified. Using Standard Reference Material (SRM) 2242, which is certified for Raman spectral correction, for monitoring the microarray fluorescence at the two most commonly used wavelengths, our team at the National Institute of Standards and Technology (NIST) has developed a method to establish scanner performance, qualifying signal measurement in microarray experiments. SRM 2242 exhibits the necessary photostability at the excitation wavelengths of 635 nm and 532 nm, which allows scanner signal stability monitoring, although it is not certified for use in this capacity. In the current study, instrument response was tracked day to day, confirming that changes observed in experimental arrays scanned are not due to changes in the scanner response. Signal intensity and signal-to-noise ratio (S/N) were tracked over time on three different scanners, indicating the utility of the SRM for scanner qualification.

INTRODUCTION

Microarray experiments, in which many thousands of signals are monitored concurrently, are becoming widely used for genome-scale measurements. For full integration of this technology into personalized medicine, users will need the ability to demonstrate the quality of their measurement results (1–5). Scanner performance, although not usually considered as a component of experimental variability or bias, has the potential to be a significant contributor to experimental results. Methods to ascertain scanner performance and qualify signal measurement in microarray experiments are under development at the National Institute of Standards and Technology (NIST). These methods are designed to track scanner performance over time and enable comparability of scanners. The method described in this paper uses a commercially available tool well characterized for use in a related technology, Raman spectroscopy, and available as a homogenous glass.

Development of a scanner qualification method requires a tool that is photostable and has adequate fluorescence signal intensity at wavelengths and instrument settings commonly used in microarray experiments. Previous studies have characterized a possible reference material composed of successive dilutions of the organic dyes cyanine 5 (Cy5) and cyanine 3 (Cy3) (6,7). As an alternative to these dyes that have been demonstrated to have limited stability, NIST Standard Reference Material (SRM) 2242, certified for y-axis Raman spectral intensity correction at 488 nm, 514 nm, and 532 nm excitation wavelengths, and known to be photostable at these wavelengths (8) (https://srmors.nist.gov/view_detail.cfm?srm=2242), was investigated. Although not certified for use in this capacity or at 635 nm, SRM 2242 exhibits the necessary photostability at the excitation wavelengths of 635 nm and 532 nm, allowing scanner signal and signal-to-noise ratio (S/N) monitoring. In the current study, the photostability of the SRM enabled tracking the instrument response

day to day, confirming that changes observed in experimental arrays scanned were not due to changes in the scanner response.

While regulations by the United States Food and Drug Administration (U.S. FDA) for analytical methods are in place requiring evidence that a specific product will meet predefined specifications (www.fda.gov/CDER/GUIDANCE/pv.htm), validation for analytical instruments, referred to as instrument qualification, is increasingly acknowledged as a fundamental component of data quality. Instrument qualification underpins analytical method validation, system suitability tests, and quality control samples, according to a recent United States Pharmacopeia (USP) chapter, USP-NF <1058>, on analytical instrument qualification (9). Using a photostable material such as the fluorescent manganese glass of SRM 2242 enables scanner qualification when the glass is used to track signal stability over time, including time periods before and after experimental scans. Ideally, the microarray scanner should respond consistently from day to day, producing signal responses that match within a predefined uncertainty. SRM 2242, with its certification for use with laser powers greater than that typically used by microarray scanners, is photostable and not subject to the degradation observed with the organic dyes typically used with experimental microarrays. The stability of SRM 2242 facilitated assessment of the microarray scanner performance separately from the performance of the material, again something not possible with most organic dyes. With a history of signal measurements using a stable material, the user can estimate the uncertainty introduced by the scanning process and be assured that the instrument variability is minimal relative to the experimental variability. Additionally, the user can be assured that the scanner performance is “in control,” and that performance of the scanner today is similar, within limits, to previous uses.

Using SRM 2242, signal intensities and S/N were compared with those of Cy3 and Cy5, with scans taken on the same days as the SRM. The signal

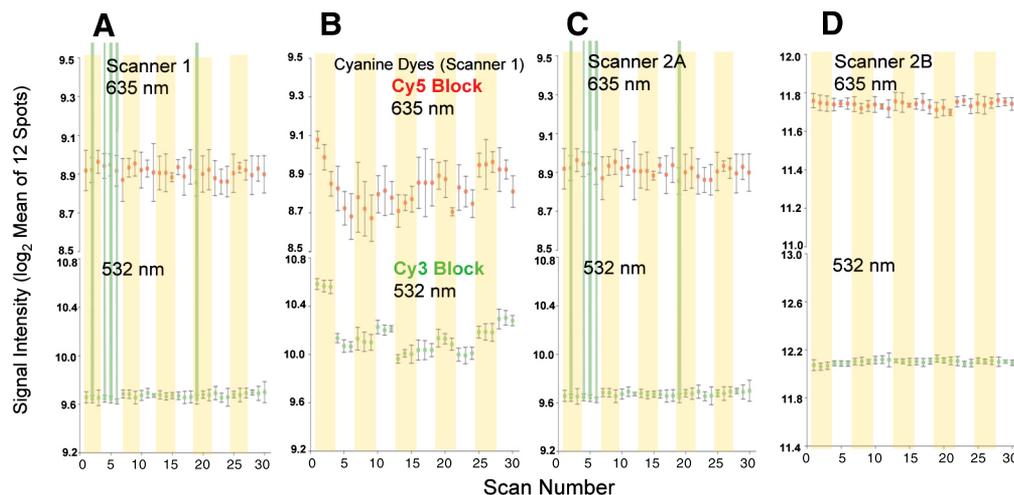


Figure 1. Comparison of signal intensity between Standard Reference Material (SRM) 2242 and the cyanine dyes, Cy5 and Cy3, on a single scanner (Scanner 1) and of SRM 2242 among three scanners. The SRM and slide with cyanine dyes were scanned in triplicate twice weekly during three different five-week time periods. Data points within a stripe represent scans taken the same day. The error bars correspond to the \log_2 median absolute deviation (MAD) of the 12 spots. The range of the signal intensities is the same on all three scanners, although the signal intensities of Scanner 2B are greater than those of Scanners 1 and 2A. Note that since the MAD is in \log_2 space it is a measure of relative variability among the measurements.

intensity and S/N of SRM 2242 were tracked over three different five-week periods on three different scanners from the same manufacturer, making scanner-to-scanner assessment possible. The stability of the signal intensity and S/N over the time periods studied is indicative of the utility of SRM 2242 for microarray scanner qualification.

MATERIALS AND METHODS

A single piece of SRM 2242 borate matrix glass (NIST, Gaithersburg, MD, USA), which was manganese-doped (0.15 wt. % MnO_2), was used throughout the study. The SRM is 10.7 mm \times 30.4 mm \times 2.0 mm. A holder the size of a microscope slide, 25 mm \times 75 mm \times 1 mm, was made to hold the SRM in place in the scanner. The holder was constructed from a polymethylmethacrylate (PMMA) sheet with a rectangle the size of the SRM glass cut out of the middle, into which the SRM was press-fit in place. Scans of the SRM were made on the smooth side of the glass; the frosted side of the glass is certified for Raman spectral correction. The smooth side of the glass was chosen for scanner measurements due to the decreased noise relative to the frosted side.

SRM 2242 was scanned three times in quick succession twice weekly over three different five-week periods, using the same instrument settings throughout the study. Triplicate scans taken on the same day are indicated by the stripes

on the plots in Figure 1 and Figure 2. On the same days that scans of the SRM were made, triplicate scans of a slide with serial dilutions of Cy5 and Cy3 dyes were acquired (Full Moon Biosystems, Palo Alto, CA, USA), but with a 5-min lag between each scan to allow for dye recovery, as recommended by the manufacturer. A single column of each dye, with 12 spots of identical concentration, was chosen for comparison to the SRM signal. Column 13 of both the Cy5 block and the Cy3 block was selected since the signal intensities of the dyes in those columns matched the signal intensities of the SRM. Data from scans of the slides with the serial dilutions of Cy5 and Cy3 dyes and SRM 2242 were not background-subtracted. Signal intensities measured on the SRM were well above the off-spot background of the cyanine dye slide, which were typically <100 units.

Three different scanners from the same manufacturer were used in this study and are referred to as Scanner 1, Scanner 2A, and Scanner 2B, with Scanner 2A and Scanner 2B being the same model. The scanners use excitation wavelengths of 635 nm and 532 nm and band pass filters of 655–695 nm and 550–600 nm, respectively. Although the instrument settings of each scanner were kept constant throughout the study, different instrument settings were used on each scanner.

Blocks of 32 columns and 12 rows with a spot size of 210 μm were

applied to both the cyanine dye slides and the SRM, although data from a single column of 12 spots were used in this study. Images were acquired as tiff files and converted to feature extracted files using software that came with the scanner (same version used throughout the study and on all three scanners). The file was exported to a spreadsheet and the signal intensities of a single column of 12 spots were averaged to produce a mean column intensity. The median absolute deviation (MAD) was used as a measure of the individual scan noise because it provides a robust measure immune to single-spot outliers. As shown in Figure 1, the geometric MAD was calculated by transforming the individual values to \log_2 values, then determining the median value of the 12 spots in the chosen column, calculating the absolute differences between the column median and each individual value, and taking the median value of the differences. To scale the MAD to the standard deviation, the final value was divided by 0.6745. Since the MAD is in \log_2 space it is a measure of relative variability. S/N measurements are the ratio of the signal means and the MAD of the 12 measurements in a column. For calculation of S/N measurements, values were not \log_2 transformed.

For calculating the variability of the signal intensities over the five weeks of the study, the relative standard deviations (RSD) of the arithmetic signal intensities of the manganese oxide glass and cyanine dye slide were calculated by dividing the standard deviation

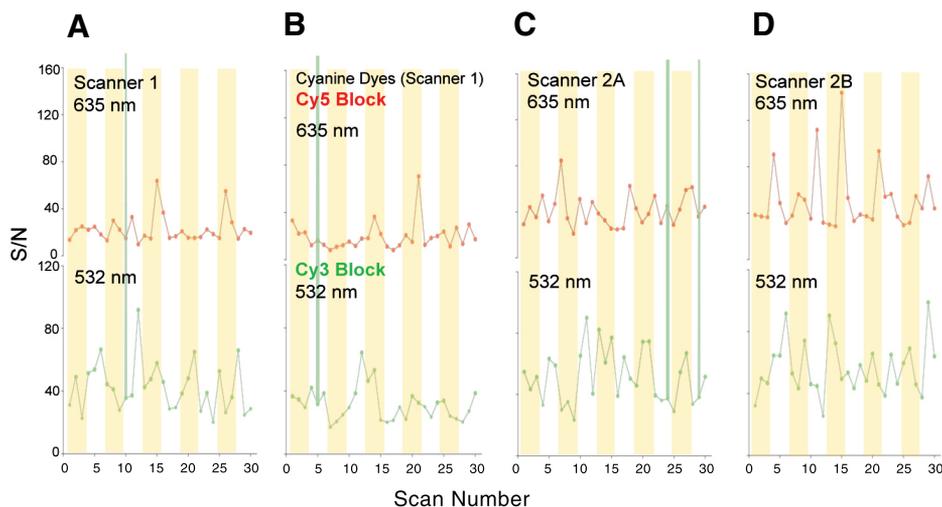


Figure 2. Signal-to-noise ratio (S/N) measurements of Standard Reference Material (SRM) 2242 on three different scanners, and of cyanine dyes, for comparison. Data points within a stripe represent data taken the same day. The signal intensities are shown in Figure 1, and the noise is the median absolute deviation (MAD) of the 12 spots in a single column of identical spots. Data used to calculate S/N measurements was not \log_2 transformed.

of the medians of each triplicate by the grand mean of all the scans over the five-week study.

To determine the homogeneity of the manganese oxide glass, a block of 35 columns by 107 rows, spot size 210 μm , was applied, covering as much of the glass surface as possible. A contour plot of the image was examined. Alternatively, the block of 32 columns by 12 rows was shifted to different positions on images of the SRM as a further assessment of SRM homogeneity.

RESULTS AND DISCUSSION

To investigate the suitability of SRM 2242 for microarray scanner qualification, experiments were carried out to compare the manganese oxide glass to the organic dyes Cy5 and Cy3, traditionally used in microarray experiments. In Figure 1, A and B, the \log_2 transformed mean signal intensities of SRM 2242 and a single concentration of the cyanine dyes on Scanner 1 are shown as tracked over the five-week period of the study. The signal stability of the SRM relative to the organic dyes is shown in Figure 3 in which box plots of the data taken throughout the study indicate the relative variability of the different materials and scanners. As a summary measure, the box plots indicate the greater overall range of the cyanine dye measurements relative to the SRM measurements. As a quantitative measure of the signal stability, the

RSDs of the signal intensities over the five weeks of the study were calculated. As expected, based on the graphical analysis, the RSDs of the SRM were considerably lower than those of the cyanine dyes; 1.6% and 1.2% at 635 nm and 532 nm, respectively, for the SRM, and 6.6% and 12.5% at 635 nm and 532 nm, respectively, for the cyanine dyes. Interestingly, although the cyanine dyes are understood to be subject to photobleaching (10), the signal variability observed was erratic and did not show a steady, consistent signal decrease as might be expected if the dyes were being permanently bleached. The instability of the dyes makes separation of scanner performance from changes in the dye difficult. A stable material is necessary to validate that the microarray scanner is working in a similar manner from day to day and scan to scan.

The photostability of the manganese oxide glass of SRM 2242 enables comparisons among scanners, as shown in this study in which the signal intensity and S/N were tracked and compared among three different scanners from the same manufacturer. The instrument settings for Scanners 1 and 2A were set based on similarity to the cyanine dye slide settings and resulted in signal intensities in the same range (unlike those of Scanner 2B), as shown in Figures 1 and 3, enabling direct comparisons. As seen in the plot of the signal intensities and in the summary plot of Figure 3, greater day-to-day variability in the signal

intensity was observed on Scanner 2A than on Scanner 1. As a quantitative measure of the variability throughout the study, the RSDs of Scanner 2A were three- to fourfold greater than Scanner 1, confirming the better signal stability of Scanner 1 at both wavelengths. Although noticeable day-to-day differences were observed between Scanner 1 and 2A, scan-to-scan differences within a single day did not differ appreciably.

While the signal variability of measurements made on Scanner 2A are greater than on Scanner 1, there was no evidence of out-of-control scans or of any instrumental or material problems during the five weeks of the study. Although signs of instrumental drift are evident in plots of the scans of SRM 2242, the instrumental variability is relatively minor, especially in comparison to the variability among the 12 spots of each individual scan. All the scanners used in this study were in good working condition, and no evidence of out-of-control events took place during the study. That being said, the better signal stability of Scanner 1 indicates that it would be more sensitive to adverse events; that is, adverse events would be easier to recognize because the range of values measured is smaller than on Scanner 2A.

Another figure of merit, S/N, was investigated for comparison among the scanners and between the cyanine dyes and SRM, as shown in Figure 2. The S/N measurements of SRM 2242 and the cyanine dyes on Scanner 1 are the most stable of the three scanners, although lower in value at 635 nm than on the other two scanners. It is worthy of note that the S/N measurements of SRM 2242 and the cyanine dyes on the same scanner are similar in intensity and similar in range, despite the different materials used. The similarity of the S/N measurements on the same

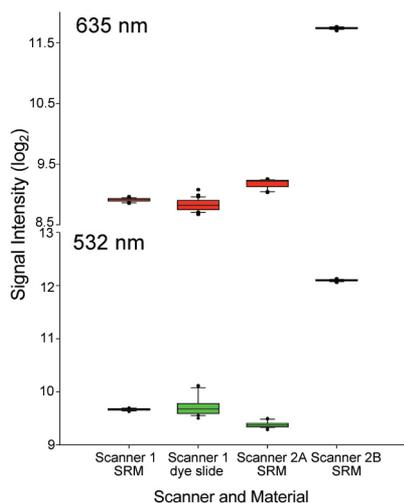


Figure 3. Summary plot of signal intensities of Standard Reference Material (SRM) 2242 and the Cy5 and Cy3 dyes. The boxes represent the two middle quartiles, the line in the box represents the median of the measurements, and the whiskers represent the 10th and 90th percentiles. Data has been \log_2 transformed.

scanner with different materials may be indicative of the utility of S/N for differentiating among scanners.

S/N measurements between the two scanners of the same model, Scanners 2A and 2B, appear similar at 532 nm although the measured signal intensities on the two scanners of the same model differed significantly (see Figure 1) due to the different instrument settings used. The spikes in S/N of SRM 2242 observed on all three scanners, especially at 635 nm, are not correlated with scan order or any other discernable factors, besides the low noise measurements evident in the error bars of Figure 1. The increased number of spikes observed on Scanner 2B at 635 nm indicates the potential usefulness of S/N as a measure of instrument performance and a potential indicator of differences among scanners.

Scans of the entire surface of the glass SRM when examined as contour plots yield information about the homogeneity of the glass confounded with the ability of the scanner to measure the same signal across a large scan area. As an additional measure of SRM surface homogeneity and scanner performance, the block of 32 columns and 12 rows was moved to different locations on the SRM, and measure-

ments were made. No significant differences were observed in signal intensity or S/N measurements when the block was placed in different positions on the SRM glass or the contour plot of the entire SRM scan examined.

As an additional measure of the effect of scanning a different area of the manganese oxide glass a study was carried out in which the SRM was left in the scanner for several days, ensuring that the same area was scanned. These experiments were compared with ones in which the SRM was removed between scan triplicates and experiments in which the SRM was removed, reinserted, and scanned again in triplicate with a minimal time lag. The six scans taken in quick succession with removal and replacement in the scanner between the first and second triplicate of scans showed the least variability. The variability associated with removing the SRM from the scanner for several days was similar to that of leaving the SRM in the scanner for similar lengths of time. Although instrumental noise is inextricably entwined with the variability associated with the SRM, based on these results instrumental noise appears to play a larger role than the SRM variability.

Use of SRM 2242 to track scanner performance over time permits monitoring two figures of merit, signal intensity and S/N. Through comparison of signal intensity and S/N over the five-week periods of the study, differences among scanners were evident. These differences highlight the importance of tracking scanner performance over time as well as the consequences of using multiple scanners for one study. Switching between scanners in the middle of an experimental study could introduce additional sources of variability that might confound the experimental results.

Performing scans of photostable qualification material such as SRM 2242 before and after experimental scans enables comparison of the signal intensity and S/N of the qualification material to previously measured values and gives the user confidence that the scanner is performing consistently throughout the sample scans. Further information on the contribution to the uncertainty of the experiment could be

determined through the use of a control chart with limits determined from assessment of qualification scans over an extended time period, work that is currently under way at NIST.

In addition to using SRM 2242 for individual instrument qualification, comparisons among scanners, as illustrated in this study, were possible in this case because the scanners had the same optical designs. Use of SRM 2242 for comparisons among scanners from different manufacturers with different optical designs may not be valid due to differing optical parameters, such as filter bandpass and measurement of depth of field. The method described here is most useful for day-to-day performance qualification and comparisons on the same instrument using the same instrument settings.

The photostability, homogeneity, and ability of SRM 2242 to produce appropriate signal intensities under conditions similar to those used experimentally make it an attractive tool for tracking scanner performance over time. The information gained from analysis of SRM 2242 will be useful in development of a tool for microarray scanner qualification with additional features, including a range of concentrations and spectral characteristics more similar to those of the Cy5 and Cy3 dyes. Such a tool would enable measurement of additional figures of merit from the calibration curve, including slope and limit of detection. The signal intensity and S/N of SRM 2242 are valuable figures of merit to track for qualification of scanner performance. As shown in this study, use of SRM 2242 in this novel application provides basic information regarding microarray signal stability and scanner performance, enabling microarray scanner qualification.

COMPETING INTERESTS STATEMENT

Certain commercial equipment, instruments, and materials are identified to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and

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Technology (NIST) nor does it imply that any of the materials, instruments, or equipment identified is necessarily the best for the purpose.

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Received 12 October 2007; accepted 12 February 2008.

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