Sequence biases in large scale gene expression profiling data

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ABSTRACT

We present the results of a simple, statistical assay that measures the G+C content sensitivity bias of gene expression experiments without the requirement of a duplicate experiment. We analyse five gene expression profiling methods: Affymetrix GeneChip, Long Serial Analysis of Gene Expression (LongSAGE), LongSAGELite, ‘Classic’ Massively Parallel Signature Sequencing (MPSS) and ‘Signature’ MPSS. We demonstrate the methods have systematic and random errors leading to a different G+C content sensitivity. The relationship between this experimental error and the G+C content of the probe set or tag that identifies each gene influences whether the gene is detected and, if detected, the level of gene expression measured. LongSAGE has the least bias, while Signature MPSS shows a strong bias to G+C rich tags and Affymetrix data show different bias depending on the data processing method (MAS 5.0, RMA or GC-RMA). The bias in the Affymetrix data primarily impacts genes expressed at lower levels. Despite the larger sampling of the MPSS library, SAGE identifies significantly more genes (60% more RefSeq genes in a single comparison).

INTRODUCTION

Gene expression profiling methods are used extensively for quantitative transcriptome analysis (1–6). Array based methods, such as Affymetrix GeneChip, rely on the hybridization of labeled transcript-derived sequences to oligonucleotide probes synthesized on the microarray. Probe hybridization intensity values are used to measure the expression levels of transcripts.

Sequencing methods, such as MPSS and SAGE generate a short sequence tag for transcripts. The gene expression level is measured by counting these tags. For 3’ tag-based methods, the ‘tag’ identified is a short stretch of nucleotides adjacent to (and including) the 3’ most site of a specific restriction enzyme in a transcript. The CAGE method is a variant that detects the 5’ most site (7). By generating tag sequences derived from an mRNA population and mapping these tag sequences to transcript databases, one can infer the abundance of transcripts in the original population. Longer tag sequences provide better specificity of mapping tags to genes. SAGE [14 bp tags; (1)] and its variants LongSAGE [21 bp tags; (2)], LongSAGELite [21 bp tags from nanogram quantities of RNA; (8)], SuperSAGE [26 bp tags; (9)] produce concatemers of tags which are cloned and sequenced using standard Sanger di-deoxy terminator sequencing chemistry. The total number of tags sequenced determines the accuracy of gene expression quantization (10) and the ability to detect rare transcripts. Recently, MPSS [20 bp tags; (4)], a sequencing technology able to generate millions of short sequences in parallel, has been used to create digital gene expression profiles at apparently lower costs than typically incurred for LongSAGE libraries sequenced using capillary sequencers and the Sanger chemistry.

New applications of gene expression profiling methods [MPSS, 5’ SAGE, poly(A)± Affymetrix tiling arrays (6,7)] are providing rich views of the transcriptome. Many papers have compared the results of different gene expression experiments across platforms and assessed platform reproducibility (11–18) without providing an explanation for the observed differences. Mecham et al. (19) and Carter et al. (20) demonstrated that incorrect assignation of Affymetrix probes to genes as one source of variability and suggested sequence-based mapping as a corrective measure. Kluger et al. (21) showed that the physical location of probes on the Affymetrix GeneChip is correlated with gene expression levels. The effect of G+C content on the stability of hybridized sequences is well known with higher G+C content corresponding to more stable DNA duplexes (22). Kuo et al. (23) found that several probe-specific factors, including G+C content, were associated with the degree of correlation between gene expression levels of the same mRNA sample.
measured by different microarray technologies. Margulies et al. (24) identified G+C content bias in 10 bp SAGE libraries and suggested a statistical analysis to assess G+C bias before deep sequencing was initiated. They gave several experimental steps for reducing bias and suggested that longer LongSAGE ditags may not suffer from the same degree of bias. Dinel et al. (25) demonstrated that SAGE replicate data was highly reproducible if one accounted for differences in concatemer and ditag replication, the number of sequenced tags and double PCR amplification of ditags.

Here, we compare and analyse publicly available gene expression data generated using one microarray method and four sequencing methods. By using a large number of publicly available experiments, we are able to measure the variation of the G+C sensitivity of all five methods by measuring the G+C sensitivity of an individual experiment without the requirement of a matched sample for comparison. We demonstrate that the variation results in an intrinsic limit on experimental reproducibility within a method (with current technology and methodology) and the ability to compare results across methods. Furthermore, we demonstrate that the differences in genes observed for both intra-method and inter-method comparisons can be explained directly in terms of the relative G+C sensitivity of the experiments.

**MATERIALS AND METHODS**

**Mapping of tags and probes to genes**

Tags were mapped to the 3’ most restriction enzyme site of transcripts using RefSeq NM genes for human and mouse (26) and the TAIR annotated gene set for Arabidopsis (27). The restriction enzyme used for the LongSAGE and LongSAGElite experiments was NlaIII (tags begin with CATG). MPSS utilized DpnII (tags begin with GATC). References to the entire set of 3’ most transcripts refer to either the set of NlaIII tags or DpnII tags, whichever is appropriate for the experiment under study.

Probes were mapped to Refseq genes using the annotation files provided by Affymetrix for the HGU 133 GeneChip (using both the A and B chips or using the A chip alone as dictated by the available data). Gene expression levels for the Human ES data were derived using both the MAS 5.0 software and GC-RMA. The gene expression values for the set of 993 experiments were derived from processed by variability of software including MAS (14). Present and absent calls from the MAS 5.0 software were used as a measure of gene presence or absence. For GC-RMA processed data, a gene expression value of 3.32 or greater was required for presence. This cutoff called present the same average percentage of genes on the chip as the MAS 5.0 software. Likewise for RMA processed data, a gene expression level of 22.1 or greater was required for presence.

**Measuring G+C bias**

For SAGE and MPSS, the bias is calculated from the premise that, for any experiment, the G+C content of the observed set of 3’ most tags should be equivalent to the G+C content of a random sampling of 3’ most tags from all transcripts. The calculation is performed in the following manner. The G+C content of the observed tags that map to the set of 3’ most tags of transcripts is determined (minus the four letter restriction site prefix). From that the deviation of the observed G+C content relative to the G+C content of the entire set of 3’ most tags is derived. To provide a fair basis of comparison that accounts for differences in the number of transcripts observed in each experiment (sampling size) as well as possible differences in the NlaIII versus DpnII tags, for each experiment, we calculate the standard deviation of the G+C content of randomly sampled tags (equivalent in number to the mapped tags that the experiment yields) relative to the entire set of 3’ most tags. The random sampling is performed 1000 times with the G+C content of the sampled tags and its deviation from the G+C content of the entire set measured each time. The standard deviation is derived from the 1000 data points. The measured deviation of the observed G+C content is divided by the standard deviation to give the number of standard deviations by which the observed G+C content varied from the expected mean. It is this measure, the number of standard deviations by which the measured mean G+C content deviates from the expected G+C content, that is used to evaluate G+C bias.

A similar approach is used for Affymetrix data. The analysis is performed at the level of the probe set. The premise is that, for any experiment, the G+C content of the observed set of probe sets that are annotated as mapping to RefSeq transcripts should be equivalent to the G+C content of a random sampling of all probe sets on the chip that are annotated as mapping to RefSeq transcripts. The rest of the calculation is performed the same way as it is for SAGE and MPSS.

Affymetrix intensity values were normalized by taking the natural logarithm of the intensity value and then subtracting the median and dividing by the interquartile range for the experiment. Signals that were reported as marginal or absent by the Affymetrix software were ignored.

**RESULTS**

**Assessment of gene coverage**

We began by assessing whether the different methods identified comparable numbers of genes. In Table 1, the first comparison shown is between a LongSAGE library of mouse liver RNA and an MPSS library derived from the same RNA sample. The MPSS library yielded 1 724 799 tags, whereas the SAGE library was sampled to only 108 117 tags. Despite the shallower depth of sampling, the SAGE library identified over 60% more RefSeq genes (3593 versus 2226; Materials and Methods). The total number of RefSeq genes that can be identified by LongSAGE with tags constructed using NlaIII (15 054) is slighter greater than that, which can be identified by MPSS with tags constructed using DpnII (14 685; Table 2). This difference is not large enough to explain the discrepancy in the number of genes identified especially considering the large difference in sampling depth. A comparison of a SAGE library and MPSS library both created using RNA purified from different adult mouse kidneys gave a similar result. In this experiment, 7291 genes were identified by SAGE and only 3547 by MPSS despite the deeper sampling by MPSS (883 305 SAGE tags...
Table 1. Differences in G+C content lead to differences in observed gene sets

| Species   | Tissue | Additional information | Technology | Library identifier | Sampling depth | Genes identified | Genes identified per sampled tag | Genes identified by both methods | Genes identified by this method only | A+T/C+G bias of tags/probes of genes missed by this method |
|-----------|--------|------------------------|------------|-------------------|----------------|-----------------|---------------------------------|----------------------------------|----------------------------------|-------------------------------|
| *M. musculus* | Liver | Same mRNA used in both experiments | LongSAGE | SM100 GSM17243 | 108 117 1 724 799 | 3593 2266 | 0.033 0.0013 | 1425 2168 | 841 | -4.31 -18.91 -0.88 8.7 |
| | Kidney | P84, male | LongSAGE | SM104 GSM34298 | 883 305 2 230 467 | 7291 3547 | 0.0083 0.0016 | 2924 4367 | 623 | -4.49 -34.06 -5.22 25.24 |
| | Visual cortex | P27, visual cortex, male | LongSAGE | SM029 SM040 | 115 803 137 562 | 5473 4702 | 0.047 0.034 | 3713 1718 | 989 | -0.32 -11.84 -7.58 10.89 |
| *A. thaliana* | Callus | Biological replicate | MPSS (classic) | CAS | 1 637 407 | 9732 | 0.0059 | 6106 3626 | 969 | 3.46 -26.31 13.2 29.78 |
| | Inflorescence | Biological replicate | MPSS (classic) | INS | 1 455 847 | 9922 | 0.0068 | 7904 2018 | 2039 | -19.39 -13.43 -11.22 25.95 |
| | Leaves | Biological replicate | MPSS (classic) | LES | 2 547 736 | 10 311 | 0.0042 | 7592 2719 | 1479 | 3.22 -23.16 11.12 30.58 |
| | Root | Biological replicate | MPSS (classic) | ROS | 3 002 218 | 9900 | 0.0033 | 7244 2656 | 1949 | -26.38 -20.68 10.01 38.27 |
| | Silique | Biological replicate | MPSS (classic) | SIS | 1 673 908 | 9715 | 0.0058 | 6157 3558 | 1216 | 7.04 -22.51 -13.05 30.63 |
| *H. sapiens* | ES | H7 p22 | Affymetrix (A/B) | WA07 a22b24 SHE13 | n/a 272 422 | 6423 | 0.024 | 3643 2780 | 2797 | 22.44 -2.08 -16.76 1.42 |
| | | | LongSAGE | SHE13 | n/a 272 | 6440 | 0.024 | 3643 2780 | 2797 | 22.44 -2.08 -16.76 1.42 |
| | | | H9 MEFs p38 | SHE98 | n/a 466 042 | 6014 | 0.016 | 3773 2241 | 3585 | 25.68 2.57 -21.22 1.11 |
| | | | H1 p54 matrigel | WA01 a20b21 SHE16 | n/a 218 169 | 6101 | 0.027 | 3375 2727 | 2519 | 25.74 -4.03 -18.11 3.13 |
| | | | H14 p22 | WA14 a23b25 SHE14 | n/a 212 136 | 6028 | 0.028 | 3326 2702 | 2694 | 24.35 2.56 -17.29 2.50 |
| | | | HES4 p36 | E804 a8b91 SHE11 | n/a 209 177 | 5940 | 0.029 | 3262 2678 | 2872 | -26.21 0.36 -19.0 3.77 |
| Species | Tissue | Additional information | Technology | Library identifier | Sampling depth | Genes identified | Genes identified per sampled tag | Number of genes identified by both methods | Genes identified by this method only | A+T/C+G library bias | A+T/C+G bias of tags/probes of genes missed by this method |
|---------|--------|------------------------|------------|-------------------|---------------|------------------|-------------------------------|----------------------------------|-----------------------------------|--------------------------------|----------------------------------|
| **Intra-method comparisons** |        |                        |            |                   |               |                 |                               |                                  |                                   |                                     |                                   |
| *M. musculus* |        |                        |            |                   |               |                 |                               |                                  |                                   |                                     |                                   |
| Heart—atria | Theiler stage 14 | LongSAGE | SM006 | 106 604 | 4600 | 0.043 | 3618 | 982 | 2.9 | -4.22 |
| Heart—bulbus cordis | Theiler stage 14 | LongSAGE | SM005 | 107 297 | 4761 | 0.044 | 1143 | -3.22 | 6.9 |
| Liver | Left lobe | MPSS (signature) | GSM32357 | 1 900 569 | 2910 | 0.0015 | 1580 | 1330 | -17.48 | -10.46 |
| | Right lobe | | GSM36724 | 1 810 280 | 2014 | 0.001 | 434 | -21.76 | -3.48 |
| Visual cortex, P27 | Biological replicate | LongSAGE-Lite | SM073 | 109 117 | 4495 | 0.041 | 3527 | 968 | -10.28 | 1.1 |
| | | | SM040 | 137 562 | 4702 | 0.034 | 1175 | -11.84 | 4.3 |
| *H. sapiens* | ES | H1 p54 matrigel | Affymetrix (A/B) | WA01 a26b30 | n/a | 6285 | n/a | 5827 | 458 | 27.91 | -3.47 |
| | H1 p54 matrigel | Affymetrix (A/B) | WA01 a21b22 | n/a | 6102 | n/a | 275 | 25.74 | 2.01 |

*aThe A+T/C+G bias of the library is measured in units of the number of standard deviations by which the observed bias deviates from neutral. A positive number indicates that the dataset is A+T rich relative to an unbiased sample. Within each pair of experiments the A+T rich dataset is always given first.

*bThis column provides the A+T/C+G bias of the tags of genes that were not observed. Consistently, the A+T rich dataset misses C+G rich tags and vice-versa.*
versus 2 230 467 MPSS tags). For both comparisons, the number of genes observed in common, 1425 genes for the first comparison and 2924 for the second comparison, were substantially reduced from the total number observed in an individual experiment, indicating that a large fraction of genes are observed in only one experiment in each comparison.

Further comparisons are provided in Table 1. Interestingly, MPSS [classic; (5)] generally identified more genes than MPSS [signature; (4)]. The numbers of genes identified by Affymetrix and LongSAGE are comparable. From Table 1 we see that, in general, the number of genes shared by a pair of experiments is only somewhat larger and often smaller than the sum total of unique genes identified by each experiment in a comparison.

These comparisons, though limited in number, demonstrated that there were differences in the number of transcripts identified using each of the methods that could not be explained by sampling depth alone. Our initial survey of transcripts observed by one method and missed by the other implicated a bias in detection sensitivity, perhaps related to the G+C content of the transcripts. Therefore, we sought to assess the G+C content bias for each experiment.

### Assessment of G+C content bias and detection sensitivity

The G+C detection sensitivity (G+C DS; Materials and Methods) was calculated for a series of gene expression profiling experiments. These were 67 Signature MPSS Mus musculus experiments [from http://www.ncbi.nlm.nih.gov/projects/geo/info/mouse-trans.html and downloaded from the Gene Expression Omnibus (28)], 83 LongSAGE M.musculus experiments (29), 21 LongSAGELite M.musculus experiments (29), 5 Classic MPSS Arabidopsis thaliana experiments (30), 12 Signature MPSS A.thaliana experiments (30), 28 Affymetrix Homo sapiens experiments (from www.transcriptomes.org), 16 LongSAGE H.sapiens experiments (from www.transcriptomes.org) and 993 Affymetrix H.sapiens experiments [from the Gene Expression Omnibus (28)]. The G+C DS is a measure of the deviation of G+C content of observed tags or probe sets from neutral and indicates increased sensitivity to G+C or A+T rich tags or probe sets.

Figure 1 shows the distribution of G+C DS among the experiments comprising each experimental group. The mean and standard deviation of G+C DS for each series of experiments is shown in Table 3. The LongSAGE data shows the least bias with a G+C DS of $0.15 \pm 3.75$. LongSAGE-Lite has some bias in detection sensitivity.

### Table 2. The number of NM RefSeq genes that can be identified by each method

| Experiment       | Species       | Number of genes that can be identified |
|------------------|---------------|----------------------------------------|
| LongSAGE (NlaIII) | Human         | 14 129                                  |
| MPSS (DpnII)     | Human         | 13 555                                  |
| Affymetrix U133A/B | Human       | 11 700                                  |
| Affymetrix U133 A | Human       | 11 193                                  |
| LongSAGE (NlaIII) | Mouse         | 15 054                                  |
| MPSS (DpnII)     | Mouse         | 14 685                                  |
| MPSS (DpnII)     | *Arabidopsis* | 22 381                                  |

For SAGE and MPPS, unique mappings are required.

Figure 1. Histogram showing the distribution of bias among five experimental methods. The A+T/G+C bias of an individual experiment is measured in units of the number of standard deviations by which the observed bias deviates from the expected bias. The biases of the individual experiments comprising each series are plotted as a histogram. The position of the peak and the width of the distribution are different for each method and illustrate differences in systematic and random error with respect to A+T/G+C bias.
towards G+C rich tags (−9.48 ± 4.89). For both *A. thaliana* and *M. musculus*, signature MPSS shows a strong G+C bias in detection sensitivity, although, for *A. thaliana*, classical MPSS shows a smaller bias towards A+T. This difference between the two MPSS methods may occur as a result of differences in the method of preparation of the samples for sequencing (30). If so, it is possible that the strong G+C bias in signature MPSS data arises in that step and not during sequencing itself. The Affymetrix data demonstrate a preferential detection of A+T rich probe sets when processed by GC-RMA and MAS 5.0. The GC-RMA software (31) uses the G+C content of probes to correct the gene expression level and Figure 1 demonstrates that Affymetrix data processed using GC-RMA has only ~70% of the bias of MAS 5.0 processed data. Unlike GC-RMA, the RMA software (32) does not correct the data for the G+C content of probes. It introduces less bias than either MAS 5.0 or GC-RMA and the bias is towards G+C rich probe sets.

To ensure that these results were not due solely to rare transcripts, we repeated the experiment restricting the analysis to transcripts observed at a level of 100 tags per million or greater for SAGE and MPSS (Supplementary Figure 1). This reduced the number of transcripts to ~17% of the original number. The smaller number of transcripts led to a larger standard deviation and hence to a smaller, measured bias for SAGE and MPSS (Supplementary Figure 1). For inter-method comparisons, the initial list of missed genes were filtered to remove those genes that the second experimental method was unable to detect because it lacked the probe set or tag. The tags or probe sets (using the second experimental method) while others were not (those with tags or probe sets with an unfavourable G+C content relative to the G+C DS of the experimental method). Having established that differences in G+C DS existed, we turned our attention to evaluating the impact of these differences on data interpretation and identification of expressed genes.

**Assessment of the impact of G+C detection sensitivity**

To assess the impact of G+C DS on gene identification, we analysed pairs of gene expression profiling experiments performed on similar samples. Where possible, we utilized pairs of experiments that constituted biological replicates, or even better, experiments performed on the same RNA sample. In a few cases, where a biological replicate was not available, it was necessary to substitute a closely matched experiment (e.g. mouse heart atrium versus mouse heart bulbus cordis at the same developmental stage). Though the lack of replicate experiments could be perceived as a limitation of our analysis, a strong, consistent pattern is demonstrated that strengthens our confidence in the observations.

Comparison of the experimental results allowed us to identify genes with detected expression using one experimental method, but not detected by the second experimental method. We refer to the undetected genes as ‘missed genes’. For intra-method comparisons, missed genes were simply those that the second experimental method failed to detect. For inter-method comparisons, the initial list of missed genes were filtered to remove those genes that the second method was unable to detect because it lacked the probe set or tag. The tags or probe sets (using the second experimental method’s tags or probe sets), corresponding to the missed genes were identified.

Using the same approach as before, we evaluated the G+C DS of the probe sets or tags associated with the missed genes (Table 1, last column). The results consistently demonstrated that if one experimental method had a more negative G+C DS relative to the second experimental method, then the tags or probe sets of genes missed by the first experiment would be A+T rich (have a positive G+C DS) and those missed by the second experiment would be G+C rich (have a negative G+C DS). For example, the first matched pair of libraries is a comparison between LongSAGE and MPSS liver libraries. The data from the LongSAGE library with a G+C DS of −4.31 are A+T rich relative to the data from the MPSS library which have a G+C DS of −18.91. Both are G+C rich relative to zero. The NlaIII tags of genes missed by LongSAGE are G+C rich (−0.88 G+C DS) and the DpnII tags of genes missed by MPSS are A+T rich (8.7 G+C DS). This result is

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**Table 3.** Mean and standard deviation of the distributions of G+C DS for each experimental series (Figure 1)

| Experiment series          | Number of experiments | Mean G+C DS | Standard deviation of G+C DS | Number of replicates required to achieve a standard error in the mean of 1.0 |
|----------------------------|-----------------------|-------------|-------------------------------|--------------------------------------------------------------------------------|
| MPSS (Signature) Mus.      | 67                    | −27.76      | 6.48                          | 42                                                                               |
| LongSAGE Mus.              | 83                    | 0.15        | 3.75                          | 15                                                                               |
| LongSAGELite Mus.          | 21                    | −9.48       | 4.89                          | 24                                                                               |
| MPSS (Classic) Arab.       | 5                     | −5.48       | 2.98                          | 9                                                                                |
| MPSS (Signature) Arab.     | 12                    | −23.7       | 5.79                          | 35                                                                               |
| Affymetrix (ES series) U133A/B | 28                  | 26.36       | 2.32                          | 6                                                                                |
| Affymetrix (ES series) U133A RMA | 28          | −7.56       | 3.54                          | 13                                                                               |
| Affymetrix (ES series) U133A GC-RMA | 28       | 18.35       | 1.96                          | 4                                                                                |
| Affymetrix (993 expts.) U133A | 993                | 21.43       | 7.62                          | 59                                                                               |
| LongSAGE Human ES          | 16                    | −0.57       | 4.07                          | 17                                                                               |
reproduced consistently for each matched pair for both inter- and intra-method comparisons. This confirms our earlier hypothesis that differences in G+C DS results in different genes observed.

Our general interpretation of these results is that both experimental methods correctly identify tags or probe sets representing transcripts in the sample; however, each method exhibits a different sensitivity to the G+C content of those transcript regions detected by it. Within each experimental method, there is a range of G+C DS, perhaps related to differences in experimental conditions. Hence, the variation in G+C DS arises from two sources: a method dependant bias (mean G+C DS; Table 3) and a method dependent variability (standard deviation of G+C DS; Table 3). The method dependant variability is related to the reproducibility of a particular method.

Looking at the LongSAGE ES Hs and Affymetrix ES Hs series (Figure 1), we see that there is variability in G+C DS for replicate samples from the same tissue and that the mean bias is similar to the mean bias of the larger distributions when they are compared to LongSAGE Mm and Affymetrix Hs series, respectively. This lends further evidence to support the assertion that the observed variance in G+C DS is a product of the technology being used to measure gene expression levels and not reflective of the real gene expression levels. The width of the larger Affymetrix series is much wider than the Affymetrix ES series. This difference is addressed in the next section.

The data in Table 1 provide additional confirmation of the G+C bias in the Affymetrix data. The comparison of LongSAGE and Affymetrix demonstrates that genes observed by LongSAGE and missed by Affymetrix have G+C rich probe sets. This orthogonal observation support the notion that the A+T bias in the Affymetrix MAS processed data reduces sensitivity of gene expression detection for genes with G+C rich probe sets.

**Variability of affymetrix results among experimental series**

Figure 1 and Table 3 show that the Affymetrix results from www.transcriptomes.org, though encapsulated within the distribution of the larger group of 993 experiments, have a much smaller standard deviation than the larger group. The ES Affymetrix data were generated by a single laboratory; hence, suspecting that different laboratories, utilizing different scanners, protocols and batches of chips, would produce data with a greater variation in G+C DS than a single lab, we divided the data into groups of experimental series as specified by GEO. Each of the chosen experimental series comprised of repeat gene expression experiments performed on the same tissue, but extracted from different individuals with the exception of GSE1296 which was comprised of gene expression experiments performed on the same tissue processed using different collection protocols.

Figure 2 provides a representative sample of the series and demonstrates that the results from each series have their own distribution. Although, there are limited data points in the individual series, the data appear to confirm our hypothesis that the different laboratories produce data with different levels of bias. The standard deviations of these distributions are smaller and hence experiments performed as part of the same series will have a higher degree of reproducibility than when compared between series. Therefore, as demonstrated previously by others (33) we expect the results of replicate Affymetrix experiments performed on the same material at different laboratories to have greater variability than those performed at the same laboratory.

![Figure 2](image.png)

**Figure 2.** Histogram showing the distribution of bias among individual series of Affymetrix experiments. For each series, the position of the peak and the width of the distribution are different. The figure illustrates individual laboratories have their own bias and variation in bias. Thus, combining experiments from different laboratories leads to a greater width in the summed distribution (Affymetrix 993 experiments). The series identifiers in the figure (e.g. GSE994) are GEO dataset identifiers.
DISCUSSION

The mean and standard deviation of the G+C DS (Table 3) can be discussed in terms of accuracy and reproducibility, respectively or systematic error and random error, respectively. A method with a larger standard deviation in observed G+C DS has more variation in experimental results. This variation can be corrected through the use of replicates, though methods with a larger standard deviation will require additional replicates to obtain a more precise measure of gene expression levels. However, replicates will not improve the accuracy of the measurement if there is a systematic error underlying the experimental method as measured by the deviation of the mean G+C DS from zero. Hence, the better method of measuring gene expression has both a G+C DS mean and standard deviation of zero. In reality, each method has its own strengths and weaknesses. While LongSAGE has the lowest mean G+C DS, replicates are relatively expensive at this time. Affymetrix has a similar standard deviation in G+C DS to LongSAGE and replicates are less expensive. For Affymetrix, the mean G+C DS is strongly related to the data processing method (MAS, RMA and GC-RMA). Although LongSAGELite demonstrates a G+C DS bias, the method allows the creation of SAGE libraries from nanogram quantities of RNA. Signature MPSS shows the strongest G+C DS bias, but allows deep profiling of RNA samples. In contrast to the results for Signature MPSS, Classical MPSS shows a much smaller bias.

The Affymetrix data show different biases with each of the data processing methods. RMA does not utilize the probeset sequence to correct the gene expression levels and that data show a G+C bias. GC-RMA attempts to correct for stronger hybridization to G+C rich probesets. Our results indicate that GC-RMA may be overcorrecting the data and an intermediate correction may yield better results.

Series of experiments performed across different platforms often utilize a reference experiment against which other experiments are normalized. This strategy is an attempt at removing the measurement biases inherent in each platform. The effectiveness of this strategy is limited by experimental realities: unknown non-linear signal response, signal saturation and background noise. For example, if a gene is observed at levels comparable to the background noise, its true expression level cannot be recovered. We have shown that the difference in detected genes is extremely large. For example, in Affymetrix MAS processed data and LongSAGE comparisons only approximately one-half of the observed genes are common to both experiments. These differences cannot be resolved by normalization and we present, for the first time, an explanation for these differences.

This study has also demonstrated that the impact of the G+C bias is not merely restricted to detection but affects the gene expression level itself. Hence, one cannot infer that gene A is more highly expressed than gene B from the gene expression intensity value assigned to each gene. While relative expression changes between individual experiments within a single platform are valid, we have shown, for the first time, that certain genes will be harder to observe (i.e. have lower expression intensity values) in certain platforms (e.g. MPSS is poor at detecting genes with high A+T content probe sets).

We have demonstrated that differences in sensitivity directly impact the genes that are observed. This observation presents an explanation and means of quantifying one source of experimental error in gene expression experiments. Of the methods studied, LongSAGE performs the best, while MPSS shows a strong bias to G+C rich tags and Affymetrix shows different biases as a function of the data processing method. The Affymetrix bias is partially corrected by the RMA software and a similar approach may improve MPSS data. Still, large experimental biases remain and an understanding of them is necessary for correct interpretation of gene expression data.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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Conflict of interest statement. None declared.

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