An antibody-based microarray assay for small RNA detection

Zonglin Hu, Aixia Zhang1, Gisela Storz1, Susan Gottesman2 and Stephen H. Leppla*

Bacterial Toxins and Therapeutics Section, National Institute of Allergy and Infectious Diseases, 1Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development and 2Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

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ABSTRACT
Detection of RNAs on microarrays is rapidly becoming a standard approach for molecular biologists. However, current methods frequently discriminate against structured and/or small RNA species. Here we present an approach that bypasses these problems. Unmodified RNA is hybridized directly to DNA microarrays and detected with the high-affinity, nucleotide sequence-independent, DNA/RNA hybrid-specific mouse monoclonal antibody S9.6. Subsequent reactions with a fluorescently-labeled anti-mouse IgG antibody or biotin-labeled anti-mouse IgG together with fluorescently labeled streptavidin produces a signal that can be measured in a standard microarray scanner. The antibody-based method was able to detect low abundance small RNAs of Escherichia coli much more efficiently than the commonly-used cDNA-based method. A specific small RNA was detected in amounts of 0.25 fmol (i.e. concentration of 10 pM in a 25 μl reaction). The method is an efficient, robust and inexpensive technique that allows quantitative analysis of gene expression and does not discriminate against short or structured RNAs.

INTRODUCTION
DNA microarrays are powerful tools that measure the expression of tens of thousands of genes simultaneously (1,2). Microarray systems have been widely used in almost every area of biological research, from basic research to clinical diagnostics (3). One of the most challenging aspects in the use of microarrays to analyze gene expression is the preparation and labeling of the RNA transcripts. Frequently, only small amounts of the biological samples are available, making capturing an accurate representation of labile RNAs difficult. Even more challenging can be detecting small, non-coding RNAs. These RNAs have been found recently to have unanticipated regulatory roles, and the study of such RNAs has taken on new importance (4,5). Many of these RNAs are very small, most being 40–300 nt in bacteria. MicroRNAs, an abundant class of small, non-coding RNA in eukaryotes, are even smaller, generally only 22 nt (4,6,7). They may be expressed under restricted conditions, can be short-lived, and may have complex secondary structures. Their small size and structure make them particularly poor substrates for cDNA synthesis using random primers; direct labeling of the RNA by ligation or chemical modification may also be impeded by their structure.

In prior work from this laboratory, a novel microarray protocol was used to identify a number of previously unknown small Escherichia coli RNAs (sRNAs) that bind the RNA chaperone protein Hfq (8). RNA isolated after co-immunoprecipitation with Hfq was hybridized to microarrays and the resulting hybrids were detected with an antibody specific to DNA/RNA hybrids. The antibody was from the Hybrid Capture ExpressArray Kit obtained from Digene Corporation (Gaithersburg, MD). Unfortunately, this kit is no longer being marketed. Because this approach showed considerable promise for the discovery and expression analysis of sRNAs, we attempted to develop a similar antibody-based strategy for detection of DNA/RNA hybrids. Here we describe an antibody-based microarray assay for DNA/RNA detection and gene expression analysis that provides simple, rapid, highly sensitive and reproducible quantitative detection of gene expression.

MATERIALS AND METHODS
Total RNA
Total E.coli RNA was purchased from Ambion (made from DH5α cultures harvested during the log phase of growth at an A600 of 0.8, catalog no. 7940, Austin, TX) or isolated from exponentially-growing cultures of MG1655 (A600 of 0.4) left untreated or exposed to 0.2 mM hydrogen peroxide for 5 min...
or overnight cultures of MC4100 cells using the hot-phenol extraction method as described previously (9). For analysis using Affymetrix arrays, the total RNA isolated from MC4100 cells was treated with Turbo DNase (Ambion) to remove residual chromosomal DNA. The 16S and 23S ribosomal RNAs (rRNAs) were also removed from one sample using MICROBEExpress Bacterial mRNA Enrichment Kit (Ambion). Total RNA and rRNA-depleted RNA were then fragmented by incubating in 1× NEB buffer for T4 polynucleotide kinase (70 mM Tris–HCl, 10 mM MgCl₂, 5 mM DTT, pH 7.6, New England Biolabs, Ipswich, MA) at 95°C for 30 min.

**Co-immunoprecipitated RNA**

RNA that co-immunoprecipitates with Hfq was prepared as described previously with the following modifications (8). Cell extracts were made from MC4100 cells grown overnight in Luria–Bertani medium at 37°C, and immunoprecipitations were carried out using 20 μl of Hfq antiserum (10), 24 mg of protein A–Sepharose (Amersham Biosciences, Piscataway, NJ) and 200 μl of cell extract per immunoprecipitation reaction. Immunoprecipitated RNA was isolated from protein A–Sepharose beads by extraction with phenol:chloroform:isoamyl alcohol (50:50:1), followed by ethanol precipitation.

**OxyS RNA**

Fragments carrying a T7 promoter and the oxyS coding sequence were amplified from plasmid pGSO100 (11) by PCR using primers (5’-CTT GAA TTC TAA TAC GAC TCA CTA TAG GGA AAG CGG GCC GCA CC and 5’-TAC AAG CTT GCG GAT CTT GGA GAT CCGCAA AAG TT). OxyS RNA then was synthesized by in vitro transcription with T7 RNA polymerase (New England Biolabs).

**Antibodies**

The mouse monoclonal antibody S9.6 directed to DNA/RNA hybrids (12) was initially provided by Dr James G. Lazar (Marigen Biosciences, Inc., Jamsville, MD), and later was produced from the hybridoma cell line purchased from ATCC (cell line ATCC HB-8730; Manassas, VA). Polyclonal antibodies to DNA/RNA hybrids (13,14) that were kindly provided by Dr B. David Stollar (Tufts University) included goat 4 A-E purified IgG, goat 4H antiserum, and sheep 4B antiserum.

Secondary antibody detection reagents included Cy3-labeled goat anti-mouse IgG (catalog no. 078-18-061; KPL, Gaithersburg, MD), Cy3-labeled rabbit anti-goat IgG (catalog no. 81-1615; Zymed Laboratories, San Francisco, CA), and biotin-labeled rabbit anti-mouse IgG (Zymed catalog no. 81-6740). Detection was carried out using streptavidin R-phycocerythrin (SAPE) conjugate (catalog no. S-866; Molecular Probes, Eugene, OR) and Streptavidin Alexa Fluor 633 conjugate (catalog no. S-21375, Molecular Probes).

**Glass slide microarray design and fabrication**

Amino-modified (Amino-C6) oligodeoxynucleotides (Supplementary Table S1) were synthesized at 0.2 μmol scale by Operon Biotecnologies, Inc. (Germantown, MD). Except for the yeast histidine-tRNA oligonucleotides, all oligonucleotides used here correspond to sequences of *E.coli* rRNA or small regulatory RNAs that have been studied previously in this laboratory (8). Oligonucleotides were dissolved in phosphate-buffered saline (PBS) (1.7 mM KH₂PO₄, 5.2 mM NaHPO₄ and 150 mM NaCl) and printed onto epoxy-coated slides (catalog no. 40042; Corning, Acton, MA) at 25 pmol per 0.5 mm diameter spot using an OmniGrid printer (GeneMachine, Ann Arbor, MI). Four identical blocks were printed on each slide, and in each block every oligonucleotide was printed twice, side by side, arranged in 6 rows and 16 columns. Prior to RNA hybridization, slides were treated with 5× SSC, 1% BSA, 0.2% SDS at 45°C for 60 min. The slides were then washed twice with water, twice with isopropanol, and air dried.

**Glass slide microarray hybridization with antibody staining**

Various amounts of RNA were added to 50 μl of hybridization buffer (HB) (100 mM MES, pH 6.6, 1 M NaCl, 20 mM EDTA and 0.01% Tween 20) supplemented with 0.1 mg/ml herring sperm DNA and 0.5 mg/ml gelatin, heated to 98°C for 5 min and placed onto the slide. The RNA solution was confined to an area of 25 × 44 mm by use of an elevated coverslip (LifterSlip, 25X44I-2-4775; Erie Scientific, Portsmouth, NH), and the slide was incubated at 45°C in a microarray hybridization chamber (catalog no. 2551, Corning) for 16 h. The coverslip was removed and the array washed several times by placing it into 50 ml conical tubes containing 40 ml of non-stringent wash buffer (NSWB) [6× SSPE (20× SSPE: 3.6 M NaCl, 0.2 M NaH₂PO₄, 0.02 M EDTA, pH 7.4, 0.01% Tween 20]. The slide was placed into a 50 ml conical tube containing 40 ml of stringent wash buffer (SWB) (100 mM MES, pH 6.6, 25 mM NaCl, 0.01% Tween 20) at 45°C for 15 min with occasional shaking, followed by another 15 min wash in SWB at 45°C in a new tube. All subsequent operations were performed at room temperature. The array slide was further washed in a 50 ml conical tube containing 40 ml of NSWB and briefly air-dried. Mouse monoclonal antibody (or other anti-DNA/RNA hybrid primary antibody) was diluted in PBS with 0.05% Tween 20 (PBSB) containing 2 mg/ml gelatin (catalog no. 170-6537; BioRad, Richmond, CA) and applied to the slide under a new LifterSlip. After 1 h incubation in a humidified box, the slide was washed successively in four 50 ml tubes, each containing 40 ml PBSB. Slides were then incubated with a fluorescently-labeled secondary antibody (or other combinations of secondary detection reagents) for 1 h as above. Finally, the slides were washed five times in 50 ml tubes containing 40 ml PBST, centrifuged 5 min at 100 g to remove all liquid, and scanned in either an Axon GenePix 4000B or 4100A fluorescence scanner, using filters appropriate for the particular fluorescent conjugate. Data were collected and analyzed using GenePix Pro 6.0 software.

**Glass slide microarray hybridization of labeled cDNA**

cDNA synthesis and fluorescent labeling was performed by combining DNase-treated total RNA (8 μg in 15 μl) with 1 μl random hexamer (catalog no. 27-2166-01; Amersham Pharmacia). The mixture was heated at 70°C for 5 min and cooled on ice. RT–PCR was done by adding 10 μl Master Mix B (100 mM DTT, 100 mM dATP, 100 mM dCTP, 100 mM dGTP and 100 mM dTTP), 2 μl dUTP-Cy3 (catalog no. PA53022; Amersham Pharmacia), 1.5 μl Superscript II
Optimization of conditions for use of antibodies for RNA detection

In preliminary experiments not detailed here, the antibody reagents were titrated to determine the optimum concentrations needed to achieve high sensitivity while maintaining specificity. Most experiments using the glass slide arrays employed two-layer sandwiches: mouse monoclonal antibody S9.6 followed by Cy3-labeled anti-mouse IgG. Although the abundant 16S and 23S rRNAs in the total \textit{E.coli} RNA sample could be detected with these arrays using as little as 10 pM of monoclonal S9.6 (data not shown), we wished to find conditions suitable for detection of the less abundant sRNAs. Therefore, we used three sRNAs in titration experiments. These showed that fluorescence intensities increased when higher concentrations of S9.6 were used in reactions followed by reaction with Cy3-labeled anti-mouse IgG (data not shown). A concentration of about 2 µg/ml S9.6 was optimum. A similar dilution series was used to select an optimum concentration of the Cy3-labeled anti-mouse antibody (Supplementary Figure S1). Based on these data, we selected 1–2 µg/ml of the Cy3-labeled antibody as optimum.

Because a stronger signal might be achieved through the amplification that occurs with the use of a three-layer sandwich, we compared the two-layer sandwich protocol to one that used antibody S9.6, followed by biotin-labeled anti-mouse IgG, and finally streptavidin conjugates of either Alexa Fluor 633 or R-phycoerythrin (Supplementary Table S2). Neither of the alternative, more complex protocols was clearly superior to the two-layer procedure. However, a three-layer sandwich was used for the high density Affymetrix arrays to accommodate the standard settings for the Affymetrix fluidics station.

RESULTS AND DISCUSSION

Identification of antibodies specific to DNA/RNA hybrids

This work began as an attempt to extend a technique that successfully identified small \textit{E.coli} RNAs using antibody detection of DNA/RNA hybrids on microarrays (8). Unfortunately, the Digene kit from which the antibody was obtained is no longer available. Although details are incomplete, the Digene kit appeared to use a goat polyclonal antiserum specific to RNA/DNA hybrids. In order to develop a comparable method, we sought other sources of antibodies having this specificity. Polyclonal sheep and goat antibodies raised against synthetic homopolymer hybrids (13) were kindly provided by David Stollar. We also became aware of a mouse hybridoma cell line, S9.6, which was developed in 1986 at Miles Laboratories (12) and is now available from ATCC. This hybridoma was produced by immunization with a duplex produced by RNA polymerase transcription of the \textit{ΦX174} bacteriophage single-stranded DNA genome. The S9.6 monoclonal antibody was shown to bind to DNA/RNA hybrids in a largely nucleotide sequence-independent manner and with a Kd of 1.2 × 10^{-11} M. In spite of the unique properties of this antibody, it appears to have been rarely used, with the last reported use being in 1992 (15).

Initial tests with the polyclonal goat and sheep antisera and the monoclonal S9.6 antibody on spotted arrays suggested that...
the greatest signal to noise and highest signals were found with the S9.6 monoclonal antibody. Because of these results, and because of the obvious advantages of monoclonal antibodies, no further effort was made to characterize or optimize the polyclonal antisera. All subsequent work used the S9.6 antibody.

Comparison of monoclonal antibody and cDNA methods for RNA detection

To characterize the antibodies described above, we constructed small spotted microarrays with oligodeoxynucleotide probes for the sense and/or anti-sense strands of small, non-coding RNAs, mRNAs, and non-translated structural RNAs (rRNAs) (Supplementary Table S1). The sRNAs chosen were well characterized as being expressed either constitutively during exponential and/or stationary phase or in response to various stresses (4,8). The glass microscope slides were printed with four identical blocks, with each oligonucleotide deposited as adjacent duplicate spots in each block.

Two types of E.coli RNA preparations were hybridized to the microarrays. One was a commercial sample of total E.coli RNA. The other samples were isolated from bacteria we grew either with (induced) or without (uninduced) exposure to hydrogen peroxide, which is known to induce the OxyS sRNA (16). For all RNA samples, detection specificity and sensitivity using a conventional cDNA method was compared with that using the monoclonal antibody method (both methods are described in Materials and Methods). The results were similar for both types of RNA. The array signals for one set of experiments, comparing the performance of the antibody method with that of the traditional cDNA method for detection of RNAs in the commercial total RNA sample are shown in Figure 1; the quantitative results for selected RNAs are summarized in Table 1. In the table, the signal for the correct strand was subtracted from the signal for the correct strand probe. On average, the incorrect strand gave signals of 30–100 U, which can be taken as background, while the correct strand gave signals of 100–8000 (Table 1).

The advantage of the antibody approach was evident when detection of small, non-coding RNAs was considered. All eight sRNAs were detected at levels well above the background by the antibody method but only two were detected by the cDNA method. In contrast, both methods had a similar ability to detect mRNAs. Thus, under the conditions used here, sRNAs are more effectively detected using the antibody method.

Sensitivity limits and probe length dependence for antibody detection of RNAs

The commercial RNA sample contained very small amounts of the OxyS sRNA (Figure 1 and Table 1). This allowed us to examine the limits of detection by adding to this RNA sample various known amounts of in vitro synthesized OxyS RNA. The results are summarized in Supplementary Figure S2. As expected, signal intensities increased as OxyS RNA amounts increased, whereas the Spot42 signal did not change. With the 50mer probes, significant signals were detectable for as little as 0.25 fmol (10 pM OxyS RNA in a 25 µl volume). In separate analyses, we serially diluted total RNA and found that rRNA could also be detected in amounts of 0.25 fmol (data not shown).

The microarray was designed to include OxyS sRNA oligonucleotide probes of lengths of 15, 20, 25, 30 35 and 40 nt in addition to the 50mers (the size for most probes in the array). The signals for the OxyS RNA were highly dependent on probe length, with signal detected only on probes of 20 nt and longer, with the signal for 25mers being only 1/10 that for 50mers (Supplementary Figure S2, data only for 25mer and 50mer are shown). This may suggest that the epitope recognized by the S9.6 antibody is a duplex of at least 15 nt. Furthermore, the high dependence on length suggests that the simultaneous binding of both antigen-binding sites of the antibody to either the same or neighboring DNA:RNA duplexes may greatly increase the retention of antibody on the array element. Finally, the array includes a probe containing a single mismatch within the 25 nt OxyS probe. The higher signal on the 25mer probe than on the mismatch probe provides evidence for specificity of detection.

Application of the antibody detection method to high density microarrays

Many array experiments utilize commercially available arrays. E.coli arrays from Affymetrix, which contain oligonucleotide probes on both strands of the intergenic regions, were used in our previous experiments to detect small, non-coding RNAs, both with direct biotin-labeling of the RNA (17) and antibody detection using the Digene kit (8). As in the spotted arrays described above, probes on different strands are needed for RNA:DNA hybrid detection and cDNA detection. To compare the results of our current method with the results we previously obtained using the Digene kit, we probed the Affymetrix E.coli sense array with RNA isolated as before by co-immunoprecipitation with Hfq (described in Materials and Methods).

Overall, we obtained a low level of background with only a limited number of probes showing high activity (93 annotated mRNA and sRNA genes and 51 intergenic regions gave signal considered significant over background; data not shown). As shown for the selected genes listed in Table 2, strong signals were obtained for sRNAs shown previously to be present in high levels in sRNA samples that co-immunoprecipitate with Hfq (4.5S, DicF, DsrA, tmRNA and Spot42), while relatively low levels of activity were detected for sRNAs shown previously to be present in low levels in such samples (e.g. 6S RNA).

We also probed the Affymetrix sense array with total RNA that was partially hydrolyzed as described in Materials and Methods; preliminary experiments indicated that hydrolysis of the RNA improved the signal. Two observations can be made. First, the highest signals were detected for tRNA and tRNA genes (88 out of the 100 genes showing the highest signal; data not shown) even after removal of 16S and 23S rRNAs by hybridization with tRNA-specific oligonucleotides attached to magnetic beads. This observation is not surprising given the abundance of these RNAs and the sensitivity of the RNA:DNA hybrid antibodies in detecting sRNAs. On the other hand, the
number of mRNAs detected with the antibodies was less than the number detected using cDNAs made from the same RNA (data not shown), although the detection was increased with increasing amounts of total RNA, suggesting that the RNA may be limiting. Second, information about possible Hfq targets could be obtained by comparing the ratio of the signal for the co-immunoprecipitation sample and the total RNA sample (Table 2, last column). For sRNAs such as DicF, DsrA and Spot42 which require Hfq for function, this ratio is >300. In contrast, for sRNAs such as 4.5S RNA and tmRNA, the ratio is less than 1.0. This comparison is a refinement over our previous global search for Hfq-binding RNAs (8) and should allow us to identify still other sRNA candidates and their mRNA targets (A. Zhang, G. Storz and S. Gottesman, unpublished data).

CONCLUSIONS AND PROSPECTS

In this report, we describe an antibody-based microarray assay for DNA/RNA detection and gene expression analysis. Polyclonal antibodies to DNA/RNA hybrids were described a number of years ago (13) and their use on solid phases for hybrid detection was suggested at that time (14). Antibodies of this type do form the basis of successful viral diagnostics marketed by Digene Corp, but are not available for other uses, such as the one described here.

The mouse monoclonal antibody S9.6 (12) is specific for DNA/RNA hybrids, and totally lacks reactivity to single or double-stranded DNA or to rRNA. It was shown to have 100-fold lower affinity to poly(A):poly(dT) hybrids than to poly(I):poly(dC) hybrids, suggesting some degree of sequence
Table 1. Quantification of RNAs detected by antibody and cDNA methods on spotted microarrays

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Antibody-based method</th>
<th>cDNA-based method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fluorescent signal intensity ± SD</td>
<td>Fluorescent signal intensity ± SD</td>
</tr>
<tr>
<td></td>
<td>AS probe</td>
<td>S probe</td>
</tr>
<tr>
<td>sRNAs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DsrA</td>
<td>891 ± 209</td>
<td>309 ± 66</td>
</tr>
<tr>
<td>Spot42</td>
<td>6015 ± 819</td>
<td>49 ± 10</td>
</tr>
<tr>
<td>OxyS</td>
<td>558 ± 42</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>DicF</td>
<td>1182 ± 30</td>
<td>67 ± 11</td>
</tr>
<tr>
<td>RyhB</td>
<td>3709 ± 1027</td>
<td>163 ± 27</td>
</tr>
<tr>
<td>OmnA</td>
<td>1781 ± 289</td>
<td>57 ± 12</td>
</tr>
<tr>
<td>RyiA</td>
<td>1615 ± 68</td>
<td>60 ± 5</td>
</tr>
<tr>
<td>MicA</td>
<td>8044 ± 813</td>
<td>105 ± 28</td>
</tr>
<tr>
<td>mRNAs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sodB</td>
<td>403 ± 6</td>
<td>46 ± 3</td>
</tr>
<tr>
<td>fhaA</td>
<td>217 ± 19</td>
<td>31 ± 11</td>
</tr>
<tr>
<td>ompA</td>
<td>633 ± 112</td>
<td>37 ± 1</td>
</tr>
<tr>
<td>galE</td>
<td>385 ± 112</td>
<td>72 ± 48</td>
</tr>
<tr>
<td>galK</td>
<td>152 ± 20</td>
<td>46 ± 9</td>
</tr>
<tr>
<td>ftiZ</td>
<td>641 ± 84</td>
<td>35 ± 8</td>
</tr>
<tr>
<td>hisG</td>
<td>162 ± 34</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>hisD</td>
<td>115 ± 28</td>
<td>46 ± 2</td>
</tr>
</tbody>
</table>

Table 2. Antibody detection of RNA hybridized to high density microarrays

<table>
<thead>
<tr>
<th>Fluorescent signal intensity</th>
<th>Total RNA—rRNA</th>
<th>Total RNA</th>
<th>IP RNA</th>
<th>Ratio for IP/total, per μg input RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5S</td>
<td>12977700</td>
<td>21249200</td>
<td>1324900</td>
<td>0.6</td>
</tr>
<tr>
<td>DicF</td>
<td>455</td>
<td>425</td>
<td>14404</td>
<td>300</td>
</tr>
<tr>
<td>DsrA</td>
<td>3963</td>
<td>415</td>
<td>32939</td>
<td>800</td>
</tr>
<tr>
<td>tmRNA</td>
<td>11023100</td>
<td>4059300</td>
<td>31055</td>
<td>0.08</td>
</tr>
<tr>
<td>Spot42</td>
<td>905</td>
<td>341</td>
<td>47028</td>
<td>1300</td>
</tr>
<tr>
<td>6S</td>
<td>13909800</td>
<td>6568700</td>
<td>2392</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Total RNA was extracted from E.coli and half of the sample was depleted for rRNA. Both samples were subsequently fragmented. RNA was also immuno-precipitated from E.coli lysates with antisera to Hfq (IP). All samples were hybridized to Affymetrix arrays and detected with the S9.6 monoclonal antibody. Signal intensities are reported for the gene as a whole (usually 15 probes) after correction for the signal of the opposite strand, as calculated by the Affymetrix software.

The microarray method described here has a number of advantages that warrant its further development and use. It is simple, rapid, sensitive, quantitative, inexpensive, and uses publicly-available reagents. Because it does not require amplification or labeling of the RNA sample, it may not suffer the variability reported to occur with some of the widely-used microarray approaches, and in particular, densely tiled arrays having probes covering all intergenic and antisense regions of a genome.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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