Nucleic Acids Research, 2006, Vol. 34, No. 7 ***e52***
doi:10.1093/nar/gkl142

An antibody-based microarray assay for small RNA detection

Zonglin Hu, Aixia Zhang[1], Gisela Storz[1], Susan Gottesman[2] and Stephen H. Leppla*

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

Received January 14, 2006; Accepted March 16, 2006

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 A_{600} of 0.8, catalog no. 7940, Austin, TX) or isolated from exponentially-growing cultures of MG1655 (A_{600} of 0.4) left untreated or exposed to 0.2 mM hydrogen peroxide for 5 min

*To whom correspondence should be addressed. Tel: +1 301 594 2865; Fax: +1 301 480 0326; Email: sleppla@niaid.nih.gov

Published by Oxford University Press 2006.

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 MICROBExpress 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 $MgCl_2$, 5 mM DTT, pH 7.6, New England Biolabs, Ipswich, MA) at 95°C for 30 min.

Co-immunoprecipated 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 AAC GGA GCG GCA CC and 5′-TAC AAG CTT GCG GAT CCT GGA GAT CCG CAA 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 (Marligen Biosciences, Inc., Ijamsville, 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-phycoerythrin (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 Biotechnologies, 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 KH_2PO_4, 5.2 mM $NaHPO_4$ 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 NaH_2PO_4, 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 (PBST) 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 PBST. 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

(catalog no. 18064014; Invitrogen) and incubating at 42°C for 90 min. The reaction was terminated by adding 10 µl of 1 M NaOH and incubating at 70°C for 15 min, followed by addition of 10 µl of 1 M HCl. The sample was transferred to a Vivaspin 500 tube with 10K cutoff (catalog no. VS0122; Vivascience) with 400 µl TE, and centrifuged at 9100 *g* for 8 min. The retentate was washed 5–6 times in a fresh Vivaspin 500 to assure removal of nucleotides and byproducts. The retained sample was recovered in a total volume of 22 µl. The Cy3-labeled cDNA was mixed with 3 µl herring sperm DNA (10 µg/µl) and 25 µl hybridization solution (50% formamide, 10× SSC, 0.4% SDS). The mixtures were heated to 98°C for 5 min and placed onto the slide under a coverslip. The slide was incubated at 45°C in a Corning microarray hybridization chamber for 16 h. The slide was washed with 40 ml of 1× SSC, 0.05% SDS in a 50 ml tube twice for 5 min, followed by washing twice with 0.1× SSC. The slide was centrifuged at 600 r.p.m. for 5 min to dry, 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.

Affymetrix microarray hybridization and antibody staining

Microarray analysis was carried out by hybridizing RNA directly to the Affymetrix *E.coli* Sense Genome Arrays (P/N 510051; Affymetrix, Santa Clara, CA) according to the Affymetrix GeneChip® Expression Analysis Technical Manual (www.Affymetrix.com) with some modifications. This Sense Genome Array contains 15 25mer probes in each gene, as well as across intergenic regions, designed to hybridize directly to mRNAs. The RNA samples (2 µg for co-immunoprecipitated RNA and 20 µg for total RNA or rRNA-depleted RNA) were added to HB supplemented with 0.1 mg/ml herring sperm DNA, 0.5 mg/ml BSA and 50 pM of the control biotin-labeled oligonucleotide B2 in 200 µl total volume, heated to 99°C for 5 min and then incubated at 45°C for an additional 5 min before being placed in the microarray cartridge. Hybridization was carried out at 45°C for 16 h on a rotary mixer at 60 r.p.m. Following hybridization, the sample solution was removed and the array was washed in the Affymetrix Fluidics station as recommended in the technical manual. Hybridization was detected using the RNA:DNA mouse monoclonal antibody described above. The antibody was diluted to 0.02 mg/ml in staining buffer (SB) (100 mM MES, pH 6.6, 1 M NaCl and 0.05% Tween 20) with 2 mg/ml BSA (600 µl total volume), loaded on the array and incubated at 25°C for 60 min. After 10 wash cycles in NSWB, the array was incubated with 0.02 mg/ml biotin-labeled rabbit anti-mouse IgG and 0.4 mg/ml rabbit IgG (Sigma, St Louis, MO) in SB with 2 mg/ml BSA at 25°C for 60 min (600 µl total volume). After another 10 wash cycles in NSWB, the arrays were incubated with 10 µg/ml streptavidin R-phycoerythrin in SB with 2 mg/ml BSA at 25°C for 60 min (600 µl total volume). After a third set of 10 wash cycles in NSWB, the arrays were scanned in an Affymetrix laser scanner (at 570 nm with a resolution of 3 µm). Data were collected and analyzed using Affymetrix GeneChip Operations Software (GCOS 1.4).

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 *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 ones that used antibody S9.6, followed by biotin-labeled anti-mouse IgG, and finally streptavidin conjugates of either Alexa Fluor 633 or R-phycoerthryin (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 *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 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 incorrect 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).

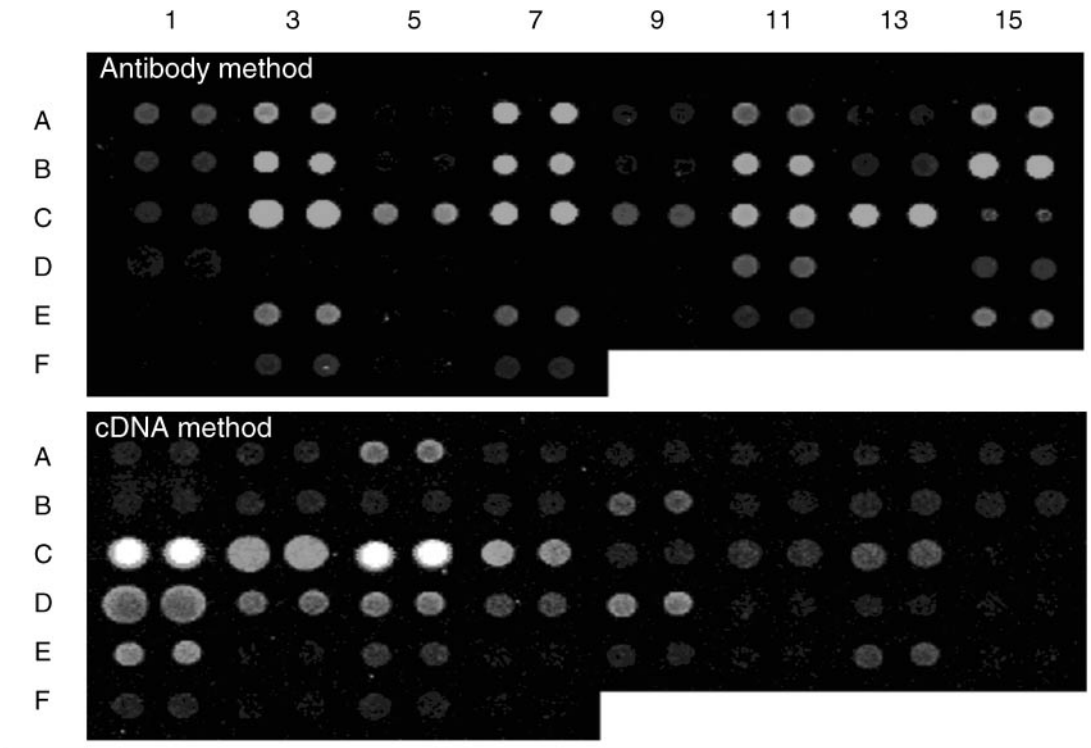
The microarray was designed to include OxyS oligonucleotide probes of lengths of 15, 20, 25, 30 35 and 40 nt in addition to the 50mers (the size for most probes in the array). The signals for the OxyS RNA were highly dependent on probe length, with signal detected only on probes of 20 nt and longer, 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 rRNA 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 rRNA-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



	1	3	5	7	9	11	13	15
A	DsrA -S	DsrA -AS	Spot42 -S	Spot42 -AS	OxyS -S	OxyS -AS	DicF -S	DicF -AS
B	RybB -S	RybB -AS	OmrA -S	OmrA -AS	RyiA -S	RyiA -AS	MicA -S	MicA -AS
C	16S rRNA -S	16S rRNA -AS	23S rRNA -S	23S rRNA -AS	His-tRNA -S (yeast)	His-tRNA -AS (yeast)	16S rRNA -AS (40 nt)	buffer
D	*sodB* -S1	*sodB* -S2	*sodB* -S3	*sodB* -S4	*sodB* -S5	*sodB* -AS	*fhlA* -S	*fhlA* -AS
E	*ompA* -S	*ompA* -AS	*galE* -S	*galE* -AS	*galK* -S	*galK* -AS	*ftsZ* -S	*ftsZ* -AS
F	*hisG* -S	*hisG* -AS	*hisI* -S	*hisI* -AS				

Figure 1. Comparison of antibody and cDNA methods for RNA detection. Spotted microarrays hybridized with 8 μg total *E.coli* RNA (Ambion) and detected by the monoclonal antibody method (top block) and cDNA method (lower block). Note that for the antibody method, signals are expected for the anti-sense spots (AS), while for the cDNA method, signals are expected for the sense spots (S). The grid at the bottom identifies the oligonucleotide in each position, with the naming conventions and sequences as shown in Supplementary Table S1. Quantification of fluorescent signals from two such arrays is presented in Table 1.

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

Probe name	Antibody-based method Fluorescent signal intensity[a] ± SD		Specific signal AS-S	cDNA-based method Fluorescent signal intensity[a] ± SD		Specific signal S-AS
	AS probe	S probe		S probe	AS probe	
sRNAs						
DsrA	891 ± 209	309 ± 66	583 ± 219	88 ± 17	111 ± 5	-23 ± 18
Spot42	6015 ± 819	49 ± 10	5966 ± 819	565 ± 184	70 ± 12	494 ± 184
OxyS	558 ± 42	92 ± 5	466 ± 43	61 ± 5	64 ± 9	-3 ± 10
DicF	1182 ± 30	67 ± 11	1115 ± 32	96 ± 14	72 ± 1	24 ± 14
RybB	3709 ± 1027	163 ± 27	3547 ± 1027	50 ± 27	145 ± 45	-95 ± 52
OmrA	1781 ± 289	57 ± 12	1724 ± 289	74 ± 15	83 ± 8	-10 ± 17
RyiA	1615 ± 68	60 ± 5	1555 ± 69	320 ± 82	60 ± 10	260 ± 83
MicA	8044 ± 813	105 ± 28	7939 ± 813	106 ± 16	74 ± 13	32 ± 21
mRNAs						
sodB	403 ± 6	46 ± 3	357 ± 7	497 ± 194	50 ± 8	448 ± 194
fhlA	217 ± 19	31 ± 11	186 ± 21	69 ± 10	29 ± 10	40 ± 14
ompA	633 ± 112	37 ± 1	596 ± 112	682 ± 208	34 ± 15	648 ± 209
galE	385 ± 112	72 ± 48	313 ± 122	177 ± 15	29 ± 10	149 ± 18
galK	152 ± 20	46 ± 9	107 ± 22	85 ± 12	24 ± 8	61 ± 15
ftsZ	641 ± 84	35 ± 8	607 ± 84	213 ± 52	16 ± 2	197 ± 52
hisG	162 ± 34	23 ± 3	139 ± 34	86 ± 31	20 ± 14	67 ± 34
hisI	115 ± 28	46 ± 2	69 ± 28	65 ± 23	17 ± 18	48 ± 29

[a]Fluorescence intensities for selected probes as measured by the array scanner for two different slides done on different days (total four spots) of which that in Figure 1 is one. AS, antisense; S, sense. Signals were averaged with SD without further correction for background. Instead, the signal of the opposite strand was taken as background to calculate a specific signal value.

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

Fluorescent signal intensity sRNAs	Total RNA—rRNA (20 μg)	Total RNA (20 μg)	IP RNA (2 μg)	Ratio for IP/ total, per μg input RNA
4.5S	12 977 700	21 249 200	1 324 900	0.6
DicF	455	425	14 404	300
DsrA	3963	415	32 939	800
tmRNA	11 023 100	4 059 300	31 055	0.08
Spot42	905	341	47 028	1300
6S	13 909 800	6 568 700	2392	0.004

Total RNA was extracted from *E.coli* and half of the sample was depleted for rRNA. Both samples were subsequently fragmented. RNA was also immunoprecipitated 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 corresponding mismatch probe, as calculated by the Affymetrix software.

preference. However, any such recognition bias did not appear to interfere with the reactivity of S9.6 to DNA/RNA hybrids formed from natural biological sequences.

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 systems (18), and our results show that it will be far more sensitive for the detection of small, non-coding RNAs of the sort found in bacteria, and possibly also for eukaryotic non-coding RNAs. Judging from the small group of sRNAs and mRNAs tested on our spotted array, we estimate that this method allows a better than 10-fold enrichment for detection of sRNAs relative to messages compared to the cDNA method. With the growing interest in sRNAs and their roles in biology, approaches such as this should be invaluable in defining these RNAs using genome-wide 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.

ACKNOWLEDGEMENTS

We thank James G. Lazar of Marligen Biosciences, Inc. and David Stollar of Tufts University for providing antibodies, NIAID core facility staff including Tim Myers, Christopher Schaupp and Guojian Jiang for slide printing and scanner assistance, Patricia Diaz of NIDCR for microarray instruction and scanner use, Nadim Majdalani, Yehoshua Gozes and Dana Hsu for advice and assistance. We thank David FitzGerald and Karen Wassarman for their comments on the manuscript. This research was supported by the Intramural Research Programs of the NIH, NIAID, NICHD, and NCI, Center for Cancer Research. Funding to pay the Open Access publication charges for this article was provided by NIAID.

Conflict of interest statement. None declared.

REFERENCES

1. Schena,M., Shalon,D., Davis,R.W. and Brown,P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science*, **270**, 467–470.
2. Lockhart,D.J. and Winzeler,E.A. (2000) Genomics, gene expression and DNA arrays. *Nature*, **405**, 827–836.
3. Heller,M.J. (2002) DNA microarray technology: devices, systems, and applications. *Annu. Rev. Biomed. Eng.*, **4**, 129–153.

4. Gottesman,S. (2004) The small RNA regulators of *Escherichia coli*: roles and mechanisms. *Annu. Rev. Microbiol.*, **58**, 303–328.
5. Storz,G., Altuvia,S. and Wassarman,K.M. (2005) An abundance of RNA regulators. *Annu. Rev. Biochem.*, **74**, 199–217.
6. Carrington,J.C. and Ambros,V. (2003) Role of microRNAs in plant and animal development. *Science*, **301**, 336–338.
7. Storz,G., Opdyke,J.A. and Zhang,A. (2004) Controlling mRNA stability and translation with small, noncoding RNAs. *Curr. Opin. Microbiol.*, **7**, 140–144.
8. Zhang,A., Wassarman,K.M., Rosenow,C., Tjaden,B.C., Storz,G. and Gottesman,S. (2003) Global analysis of small RNA and mRNA targets of Hfq. *Mol. Microbiol.*, **50**, 1111–1124.
9. Masse,E., Escorcia,F.E. and Gottesman,S. (2003) Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. *Genes Dev.*, **17**, 2374–2383.
10. Zhang,A., Wassarman,K.M., Ortega,J., Steven,A.C. and Storz,G. (2002) The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs. *Mol. Cell*, **9**, 11–22.
11. Zhang,A., Altuvia,S., Tiwari,A., Argaman,L., Hengge-Aronis,R. and Storz,G. (1998) The OxyS regulatory RNA represses rpoS translation and binds the Hfq (HF-I) protein. *EMBO J.*, **17**, 6061–6068.
12. Boguslawski,S.J., Smith,D.E., Michalak,M.A., Mickelson,K.E., Yehle,C.O., Patterson,W.L. and Carrico,R.J. (1986) Characterization of monoclonal antibody to DNA.RNA and its application to immunodetection of hybrids. *J. Immunol. Methods*,, **89**, 123–130.
13. Kitagawa,Y. and Stollar,B.D. (1982) Comparison of poly(A).poly(dT) and poly(I).poly(dC) as immunogens for the induction of antibodies to RNA–DNA hybrids. *Mol. Immunol.*, **19**, 413–420.
14. Stollar,B.D. and Rashtchian,A. (1987) Immunochemical approaches to gene probe assays. *Anal. Biochem.*, **161**, 387–394.
15. Casebolt,D.B. and Stephensen,C.B. (1992) Monoclonal antibody solution hybridization assay for detection of mouse hepatitis virus infection. *J. Clin. Microbiol.*, **30**, 608–612.
16. Altuvia,S., Weinstein-Fischer,D., Zhang,A., Postow,L. and Storz,G. (1997) A small, stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator. *Cell*, **90**, 43–53.
17. Wassarman,K.M., Repoila,F., Rosenow,C., Storz,G. and Gottesman,S. (2001) Identification of novel small RNAs using comparative genomics and microarrays. *Genes Dev.*, **15**, 1637–1651.
18. Marshall,E. (2004) Getting the noise out of gene arrays. *Science*, **306**, 630–631.