Published in final edited form as:
J Microbiol Methods. 2010 July ; 82(1): 36–41. doi:10.1016/j.mimet.2010.03.017.

Development and validation of a resistance and virulence gene microarray targeting *Escherichia coli* and *Salmonella enterica*

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Abstract

A microarray was developed to simultaneously screen *Escherichia coli* and *Salmonella enterica* for multiple genetic traits. The final array included 203 60-mer oligonucleotide probes, including 117 for resistance genes, 16 for virulence genes, 25 for replicon markers, and 45 other markers. Validity of the array was tested by assessing interlaboratory agreement among four collaborating groups using a blinded study design. Internal validation indicated that the assay was reliable (area under the receiver-operator characteristic curve=0.97). Inter-laboratory agreement, however, was poor when estimated using the intraclass correlation coefficient, which ranged from 0.27 (95% confidence interval 0.24, 0.29) to 0.29 (0.23, 0.34). These findings suggest that extensive testing and procedure standardization will be needed before bacterial genotyping arrays can be readily shared between laboratories.

1. Introduction

The characterization of antimicrobial resistance among Gram-negative pathogens may benefit greatly by identification of the genetic traits involved rather than simple determination of the resistance phenotype. In *Salmonella enterica*, for example, there are at least five genes that code for tetracycline resistance, six that code for resistance to chloramphenicol and/or florfenicol, seven that code for streptomycin/spectinomycin resistance, and thirteen for trimethoprim resistance (Michael et al., 2006). In addition to multiple known genetic determinants for each resistance phenotype, new resistance genes and mechanisms continue to be discovered (Nordmann et al., 2008, Pitout and Laupland, 2008). Transmission of

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resistance genes among bacteria is also highly diverse, involving mutations (transmitted vertically) and horizontal acquisition via plasmids, transposons, and other mobile elements (Boerlin and Reid-Smith, 2008). Because of this diversity, emergence and spread of novel resistance characteristics are difficult to monitor based solely on phenotypes. For example, resistance to fluoroquinolones in Gram-negative bacteria was largely presumed to result from point mutations in DNA gyrase and topoisomerase IV genes, but plasmid-mediated fluoroquinolone resistance genes have been identified in recent years (Cattoir and Nordmann, 2009). Very diverse families of beta-lactamase genes with similar or indistinguishable resistance phenotypes have emerged and spread globally, but the predominant genotypes may differ regionally (Bush, 2008, Canton and Coque, 2006, Pitout, 2008, Pitout and Laupland, 2008).

A microarray assay is a logical approach to screen Gram-negative bacteria for diverse and novel resistance genes, enabling simultaneous detection of hundreds to thousands of genetic elements and the flexibility to add or subtract elements as needed (Call, 2005, Call et al., 2003). It offers the potential to simultaneously identify other genes that are associated with resistance genes and that may play important roles in the epidemiology and transmission of resistance, such as integrase genes, plasmid incompatibility group markers and virulence genes. Nevertheless, the application of microarray technology for detection of resistance-associated genes must demonstrate intra-and inter-laboratory repeatability to gain acceptance as a valid diagnostic and/or screening test (Weiss, 1986).

Previously published DNA microarrays targeting resistance genes of Gram-negative bacteria have demonstrated that microarrays can be used effectively to detect and discriminate between multiple antimicrobial genes (Bruant et al., 2006, Call, et al., 2003, Chen et al., 2005, Frye et al., 2006, Frye et al., 2009, Hopkins et al., 2007, Ma et al., 2007, Majtan et al., 2007, Malorny et al., 2007, Peterson et al., , van Hoek et al., 2005, Zou et al., 2009). Although many of the probe sets published in these reports overlapped with our needs, some were limited with respect to the number and types of probes (Call, et al., 2003, Grimm et al., 2004, Lee et al., 2002, van Hoek, et al., 2005, Volokhov et al., 2003), and none of these assays had been tested for validity across multiple laboratories. Therefore, our goal was to build on these previously published arrays to target resistance and other genetic elements of Gram-negative bacteria, and to test the inter-laboratory portability of the arrays using a blinded validation study.

2. Methods

2a. Bacterial isolates

Bacterial isolates used in the receiver-operator characteristic (ROC) curve analysis and in the blinded validation study included *Salmonella enterica* and *Escherichia coli* (*E. coli*) strains with published genome sequences or strains for which the presence of specific genes had previously been demonstrated (Table 1). All bacterial species used in the study were stored in 15% glycerol at -80 °C.

2b. Phenotypic antimicrobial resistance testing

Antimicrobial susceptibility testing was conducted using a disk diffusion method (Bauer et al., 1966) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI., 2008). Bacterial isolates were tested for susceptibility using the following antimicrobial disks (potency): ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), streptomycin (10 μg), tetracycline (30 μg), sulfisoxazole (250 μg), trimethoprim-sulfamethoxizole (1.25 μg-23.75 μg), ceftazidime (30 μg), amoxicillin-clavulanic acid (20/10 μg), and nalidixic acid (30 μg) (BD Diagnostics, Sparks, Maryland, USA).

2c. Oligonucleotide probe design

The list of relevant genes for this array was developed by conducting a literature review for important Gram-negative resistance determinants, virulence genes, and replicon markers, *Salmonella*-specific markers and *E. coli* pathogen type markers (supplementary Supplementary Table 1) (Call, et al., 2003, Cassone et al., 2008, Cattoir et al., 2007, Chen, et al., 2005, Cleven et al., 2006, Frye, et al., 2006, Lee, et al., 2002, Malorny, et al., 2007, Michael, et al., 2006, Pritchett et al., 2000, van Hoek, et al., 2005). When probe sequences were not available from the published literature, they were designed using the software ArrayOligoSelector (Zhu et al., 2003). All probes were 60 nucleotides in length. Average Tm of 203 probes was 71.2°C (median 71.6, range 61.7-89.4) and average %GC was 50.8 (median 53.3, range 28.3 – 66.7). Accession numbers, sources and sequence data are available in Supplementary Table 1. The final probe list consists of 203 60-mer oligonucleotide probes, of which 117 are for bacterial resistance genes, 16 for virulence genes, and 25 are for replicon markers (Table 2).

2d. Microarray slide printing

Oligonucleotide probes (n=203, 60-mer) were printed at a 60 μM final concentration (100-500 pL per spot) onto masked-well glass slides coated with Teflon/epoxy-silane (Tekdon, Inc., Myakka City, FL) using a piezoelectric non-contact spotter (S3 SciFlexarrayer, Scienion, Berlin, Germany). The standard print buffer (2× spotting solution) from Telechem (Sunnyvale, CA) was used in the probe solution. The array was spotted using the following parameters: a 22×22 grid at 280 dot pitch, 10 grids per slide, and an average spot diameter of 200 μm. Two water washes (one 100 μL wash/flush and one 250 μL wash/flush) were carried out between each probe spotting. After printing, array slides were baked at 130°C for 1 h under a vacuum of 21 in Hg and protected from light until used. Probes were printed as duplicate spots on each array, and each sample was hybridized onto two arrays of the same slide.

2e. Genomic DNA extraction and labeling

Total bacterial DNA was extracted using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. When final DNA concentration was less than 25 ng/μl, DNA was ethanol-precipitated and resuspended in PCR grade water (50 μl) to a concentration of ≥ 25 ng/μl. Resulting bacterial genomic DNA (1 μg) was biotinylated using a nick-translation kit (BioNick, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

2f. Hybridization and detection

Biotinylated DNA (50 μl) was diluted with 2× hybridization buffer (50 μl), boiled for 3 min, and held on ice. Slides were prepared by immersing in bovine serum albumen (BSA) blocking solution (1% BSA, 3X SSC) (50 ml) for 10 min at room temperature with shaking, followed by washes in deionized water and spin-drying. Labeled genomic DNA (45 μl) was then applied to each array and allowed to hybridize for 16 - 18 h at 55°C. Hybridized slides were washed with three buffers of increasing stringency, washed three times in TNT buffer (0.1M Tris-HCL pH 7.5, 0.15M NaCl. 0.05% Tween 20) and spun dry. A tyramide signal amplification (TSA) kit (TSA Biotin System, Perkin Elmer, Waltham, MA) was immediately used in conjunction with the fluorophore SA-Alexa 555 (Molecular Probes/Invitrogen, Carlsbad, CA). The slides were scanned using an arrayWoRx Auto*e* scanner (Applied Precision, Issaquah, WA). Individual spot-intensities were annotated using softWoRx Tracker software (Applied Precision) and exported to an Excel (Microsoft, Redmond, WA) spreadsheet for analysis. A step-by-step protocol for the entire procedure from genomic DNA extraction through detection and scanning is available as a supplementary file (Appendix A, Detailed protocol for array hybridization and detection).

2g. Internal validation of array

Positive control isolates were available for 40 gene probes (Table 1). Among these, 23 probes were specific to genes known to be present for two isolates for which published sequences were available, *E. coli* O157:H7 EDL933 (Perna et al., 2001) and *Salmonella* enterica serovar Typhimurium LT2 (McClelland et al., 2001). In addition, positive array hybridization results were confirmed by PCR for 40 gene probes in six *Salmonella enterica* isolates, one *E. coli* isolate, and one *Shigella sonnei* isolate. Isolates, gene probes and PCR primers are described supplementary Supplementary Table 2. Seven isolates were assayed twice (Supplementary Table 1), *Salmonella enterica* serovar Typhimurium S2057 was assayed four times, and *E. coli* O157:H7 EDL933 (Perna, et al., 2001) was assayed five times by the same technician. These data were used to calculate Kappa statistics between two experiments and the intraclass correlation coefficient (ICC) to estimate agreement among more than two experiments.

2h. Interlaboratory repeatability study

Four collaborating laboratories with experience in microarray technology agreed to participate in the blinded study. Each laboratory (designated Lab A through D) was mailed a coded set of 5 isolates (Table 1) and the reagents specific to our protocol. The number of subjects required for an adequate sample size to estimate Kappa with 80% statistical power for a two-tailed test is 50 (Sim and Wright, 2005); Five isolates assayed with 203 probes each provided 1,015 “subjects” for the Kappa and ICC calculations. A complete list of reagents supplied to the collaborating laboratories is available as supplementary content in Appendix A. Briefly, these reagents included two array slides (each slide with 10 wells had the capacity for testing 5 samples and one extra slide was provided), the five bacterial test isolates, one kit for total DNA extraction, one BioNick kit, hybridization and detection reagents, a box and packing slip for returning hybridized slides and a detailed, illustrated protocol. Additional array slides were provided if necessary. The number code for the test isolates was established and maintained by a separate laboratory staff member and samples were only decoded after fluorescence data from collaborators had been analyzed.

2i. Data analysis

Median fluorescence intensities from four replicate spots were averaged for each probe. The resulting average fluorescence intensity values were normalized as follows: after exclusion of biotin and empty spots, the intensity of each remaining spot was divided by the average of all fluorescent intensities for all spots with values greater than 10,000 to create a normalized intensity value. To determine an optimal cutoff for a positive probe, a ROC curve was plotted using the sensitivity and specificity values generated by testing known positive and negative controls and the area under the curve (AUC) was calculated as formulated by Bewick et al. (2004). A positive probe hybridization result was considered a true positive when the result was expected because of previously established characteristics of the isolate (Table 1). All probe sequences on the array were compared to published sequences of *E. coli* O157:H7 EDL933 and *Salmonella enterica* serovar Typhimurium LT2 (Table 1) using the National Center for Biotechnology Information's BLAST alignment tool (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). All negative probe results from hybridizations with these two sequenced isolates were considered true negatives for those probes with no sequence homology to those two published genomes.

Based on the ROC analysis, a result was considered positive when normalized fluorescence intensity was greater than 0.2. To estimate overall inter-laboratory reliability, the ICC (a Kappa-equivalent statistic for multiple raters and multiple subjects) was calculated according to Fleiss (1981). For each laboratory pair the Kappa statistic and the 95% confidence interval for the Kappa statistic was calculated using SAS 9.1 (Cary, NC); all other statistics were calculated

using Microsoft Excel (Redmond, WA). Kappa and ICC were calculated for data from 1) all probe results from all five isolates, 2) only those probes for which true value (true positive or negative) were known for that isolate, and 3) all probes but excluding data from a test isolate for which one of the participating laboratories was not able to obtain a positive result for any of the probes.

3. Results

3a. Internal validation and ROC analysis

All positive array hybridization results used to develop the ROC curve were confirmed as positive using PCR (Supplementary Table 1). Ten bacterial isolates including one *E. coli* O157:H7, three *Salmonella enterica* serovar Typhimurium, two *Salmonella enterica* serovar Newport, one *Salmonella enterica* serovar Uganda and six laboratory strains containing cloned resistance genes were assayed with the array (Table 2). These assays resulted in a total of 132 known true positive and 1,353 known true negative probe tests. The AUC was calculated assuming that when 1-specificity = 1, sensitivity = 0.969 because there was no increase in sensitivity above that for the top three data points. The AUC is 0.97 (Fig. 1) indicating that overall the array provides valid results. An AUC equal to 1 would indicate a perfect test whereas an AUC less than or equal to 0.5 would indicate a test result no better than chance alone (Bewick, et al., 2004). Sensitivity intersected with specificity at a 0.20 threshold value (Fig.2) which was therefore used as the threshold to define a positive result. The Kappa statistic for agreement between within-laboratory duplicate hybridizations was 0.76 (0.69, 0.82). The ICC for 5 replicate assays of *E. coli* O157:H7 EDL933 was 0.67 (0.41, 0.92) and for 4 replicate assays of *Salmonella enterica* serovar Typhimurium S2057 was 0.83 (0.63, 1.0).

3b. Inter-rater reliability

The ICC for all four laboratories over all probes was 0.27 (95% confidence interval (CI) 0.24, 0.29). The Kappa statistic for each laboratory pair using data from all probes on the array ranged from 0.14 to 0.46. Restricting the analysis to data obtained from isolates for which all laboratories were able to obtain positive signals (the second analysis) resulted in an ICC of 0.28 (95% CI 0.25, 0.31) and Kappa statistics ranging from 0.13 to 0.47. The third analysis using probe-isolates combinations with known true values resulted in an ICC of 0.29 (0.23, 0.34) and Kappa statistics ranging from 0.11 to 0.40 for laboratory pairs (Table 3).

4. Discussion

The results of this study indicate that the resistance gene microarray developed in this laboratory had good intra-laboratory validity, as measured by the area under the ROC curve and by fair agreement between duplicate experiments. In contrast the inter-laboratory reliability after correcting for chance agreement was low as estimated by pair-specific Kappa statistics and summary intraclass correlation coefficients. While this was a disappointing finding in light of the proficiency of the four participating laboratories and in light of our efforts to provide standardized materials and support, it highlights the technical complexity of microarray technologies. Each step in array testing, including genomic DNA extraction, biotinylation using nick translation, hybridization, detection, scanning and image segmentation has inherent potential for errors and even in the most experienced hands may need troubleshooting to achieve reproducible results. The finding that intra-laboratory agreement between experiments was relatively high is not surprising because the technician was not blinded to the isolates' resistance status and because experience with the array platform had been acquired during array development. One isolate failed to yield a positive signal from any probe in one of the participating laboratories, but excluding that isolate from the analysis did not improve agreement significantly. The goal of this study was to test whether the array protocol could be

validated for transfer between laboratories. Our findings indicate that acceptance of a genotyping microarray as a standard diagnostic tool is unlikely unless the variations in results due to technical complexities are addressed.

Publications describing development and use of bacterial genotyping arrays, including those targeting resistance determinants, primarily assert their validity based on test hybridizations using control isolates (often isolates with available genome sequences data) and/or by confirming positive hybridization results with specific PCR assays (Bruant, et al., 2006, Call, et al., 2003, Cleven, et al., 2006, Hopkins, et al., 2007, Lee, et al., 2002, Majtan, et al., 2007, Martinez et al., 2006, Perreten et al., 2005, Peterson, et al., , Spence et al., 2008, van Hoek, et al., 2005, Volokhov, et al., 2003, Yu et al., 2004). But laboratory sensitivity and intra-rater reliability are only part of the validity of a diagnostic test. None have reported a blinded study to examine inter-laboratory portability in spite of claims to having diagnostic utility (Lee, et al., 2002, Martinez, et al., 2006, Peterson, et al., , Yu, et al., 2004, Zhu et al., 2007) and/or usefulness as a screening or surveillance test (Bruant, et al., 2006, Cassone, et al., 2008, Chen, et al., 2005, Volokhov, et al., 2003). In contrast, those in the field of differential gene expression array studies have done a great deal of work to address cross-laboratory and cross-platform reliability (Canales et al., 2006, Chen et al., 2007, Fan and Niu, 2007, Fan et al., 2009, Guo et al., 2006, Kadota et al., 2009, Mao et al., 2009, Sato et al., 2009, Shi et al., 2008, Shi et al., 2008, Shi et al., 2006, Shi et al., 2005, Tong et al., 2006) as well as data consistency (Brazma, 2009). For example, the MicroArray Quality Control (MAQC) project (Shi, et al., 2006) was initiated by the US Food and Drug Administration in response to scientific publications that raised concerns about reproducibility of microarray data (Shi, et al., 2008). Several publications of MAQC data indicated high inter-laboratory correlation between gene expression array results (Chen, et al., 2007, Mao, et al., 2009, Sato, et al., 2009, Shi, et al., 2008); fewer assessed agreement between laboratories with regard to qualitative results, i.e. whether or not a gene was differentially expressed. Among those, this ranged from 65% to 98% agreement (Mao, et al., 2009, Shi, et al., 2006). This was better than the agreement reported in an earlier publication by Irizarry *et al*. in which inter-laboratory within-platform agreement on gene lists ranged from 10% to 65% for a five-laboratory study (Irizarry et al., 2005). Reasons for the difference between the MAQC and Irizarry results, and between the MAQC results and ours are difficult to identify. The MAQC data were based on two reference mRNA samples for which each site performed five replicates, while our study had a more diverse set of test isolates. In addition, the highest reported concordance was for “discriminating genes” only (Mao, et al., 2009), which may have contributed to a favorable result. Finally, there was no indication that the participants were blinded as to the status of the samples.

The purpose of the present study was to develop a useful tool for epidemiologic analysis of antimicrobial resistance determinants in gram-negative bacteria, and to test its inter-laboratory reliability. The ROC curve provided evidence that this array is a potentially useful tool. Our findings of low concordance between laboratories indicate that this and other bacterial genotyping arrays should be tested rigorously across laboratories before being widely adopted to aid clinical and public health decision-making.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge the technical assistance of Stacey LaFrentz, Katherine N.K. Baker and Dennis M. Satterwhite. This project has been funded in whole with federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract numbers N01-A1-30054 and N01-A1-30055.

Appendix A

Protocol for blinded evaluation of resistance gene microarray

As part of the NIH FWD-IRN-funded project entitled “Characterization of bacterial antimicrobial resistance using a validated microarray”, a blinded study will be carried out in which five collaborating institutions will be asked to assay a set of isolates using the printed microarray slides from this project. The WSU ZRU has a set of twenty bacterial isolates with a total of 55 resistance genes among them. A WSU microbiologist located in a separate lab from the principle investigators will maintain this set of isolates, assign each one a random number, and assemble coded isolate sets for each collaborating institution.

WSU ZRU staff will assemble and mail to each collaborating institution:

1. Seven printed microarray slides. Each masked-well slide has 10 wells. Eight of the ten wells contain arrays that will be used in the protocol. Each sample will be hybridized in duplicate (in two wells) on a slide.
2. Twenty coded positive-control isolates as glycerol stocks.
3. 1 DNeasy tissue kit for genomic DNA extraction (50 reactions).
4. 1 BioNick kit (50 reactions).
5. Hybridization and detection reagents and protocols.
6. A box and packing slip for shipping slides back
7. An overall protocol with illustrations for each step of the procedure.

Collaborating institutions will

1. Streak for isolation on LB plates.
2. Grow isolates in LB broth.
3. Extract gDNA and concentrate if necessary.
4. Nick-Translate and re-suspend gDNA.
5. Perform the hybridization protocol and scan the hybridized slides.
6. Export and save fluorescence intensity (or equivalent) data to be analyzed at the WSU ZRU as well as the digital images of the slides.
7. Return hybridized slides to the WSU ZRU in the box provided. These slides must be numbered with the appropriate code so they may be linked to the correct isolates.

Culturing isolates and harvesting cells

Keep in mind you need to be using BSL 2 procedures with all of these isolates —You will need:

LB agar

LB broth

Culture tubes

Inoculating loops

Orbital shaker

Incubator set to 37°C

Pipettes, tips, serological pipettes and pipette aid

1.5ml micro-centrifuge tubes

Table-top centrifuge

1. Streak for isolation on LB plates (see recipe) from the glycerol stocks. Incubate overnight @ 37°C.
2. Transfer an isolated colony to a culture tube containing 3 ml LB broth (see recipe) and incubate overnight at 37°C shaking at 200 rpm.
3. Transfer 1 ml broth culture to micro-centrifuge tube. Pellet at 4000 rpm for 10-15 minutes and pour off supernatant. Repeat until all broth culture has been pelleted.

Genomic DNA extraction

DNA extraction

Qiagen DNeasy silica-gel adsorption method—This protocol follows the instructions provided by the Qiagen Dneasy Tissue Kit (Qiagen, Valencia, CA; Cat. No. 69504). DNA must be quantified to properly scale the subsequent nick translation and should have A_{260}/A_{280} ratio of 1.7 to 2.

You will need:

Pipettes and tips

Table-top centrifuge

Qiagen DNeasy kit

100% ethanol (absolute)

RNase A 100mg/ml

55°C waterbath

70°C waterbath

1.5 micro-centrifuge tubes

Spectrophotometer

1. Resuspend cells from step 3 in 180 μL of buffer ATL from the Qiagen DNeasy kit.
2. Add 20 μL of Qiagen proteinase K solution and mix by vortexing.
3. Incubate at 55°C for 1-3 hours (until the suspension clears). Vortex for 15 seconds every 20 minutes.
4. Add 20 μL of RNase A (100mg/mL) to each tube, vortex, and incubate at room temperature for two minutes.
5. Vortex and add 200 μL of buffer AL, vortex, and incubate at 70°C for 10 minutes.
6. Add 200 μL of 100% ethanol. Vortex for 15 seconds.
7. Pipette the treated lysate into a DNeasy column in a collection tube, and centrifuge for 1 minute at 10,000×g.
8. Discard collection tube and put the column in a new collection tube.
9. Add 500 μL AW1 and centrifuge for 1 minute at 10,000×g.

10. Repeat step 11. Wash the column with 500 μL AW2. Centrifuge at max speed for 3 minutes to ensure the column is dry.
11. Place the columns into a 1.5 ml micro-centrifuge tube.
12. Add 200 μL AE buffer. Incubate the columns at room temperature for 1 minute and elute by centrifuging for 1 minute at 10,000 × g.
13. Quantitate the DNA using a spectrophotometer.

Ethanol Precipitation

Concentration of DNA

Ethanol precipitate the DNA if the concentration is less than 25 ng/ μL. A total of 1 μg DNA is needed in 40 μl. The DNA should have A_{260}/A_{280} of 1.7 to 2.0.

You will need:

3M sodium acetate, pH 5.2

100% ethanol (absolute)

70% ethanol

Pipettes and tips

Centrifuge

Paper towels

Vacuum centrifuge (Speed Vac)

Nuclease free water

Spectrophotometer

1. Add 1/10 volume of 3 M sodium acetate (20 μL) to the 1.5 ml micro-centrifuge tube containing the DNA needing to be concentrated.
2. Add 2 volumes of 100% ethanol (440ul).
3. Mix by inversion 10×.
4. Incubate at -80 for 30 minutes.
5. Centrifuge at max speed for 30 minutes at 4 °C.
6. Carefully decant off supernatant and blot the tube on paper towels.
7. Add 400ul 70% ethanol and pipette to resuspend.
8. Repeat steps 5-6.
9. Dry pellets with a vacuum centrifuge for 5-10 minutes, until pellet is dry, or air dry.
10. Resuspend the DNA with 50ul water.
11. Quantitate the DNA using a spectrophotometer.

Nick Translation

Biotinylation and fragmentation of DNA

You will need:

Invitrogen Nick Translation kit

Pipettes and tips

0.2 ml PCR tubes

1.5 ml micro-centrifuge tubes

Thermal cycler

Container with ice

3 M sodium acetate, pH 5.2

100% ethanol (absolute)

70% ethanol

Centrifuge

Paper towels

Vacuum centrifuge (Speed Vac)

Nuclease free water

1× hybridization buffer

1. Combine the following in 0.2ml PCR tubes on ice:

Use a total of 1 μg of DNA in a total volume of 40ul.

1 μg (up to 40ul) DNA

5 μL 10× dNTP mix (from Nick Translation kit)

5 μL 10× enzyme mix (from Nick Translation kit)

total volume of 50μL

2. Incubate the mixture at 16°C in a thermal cycler for 2 hours and then hold at 4 °C.

3. Transfer the samples to 1.5 ml micro-centrifuge tubes.

4. Ethanol precipitate nick translated DNA-

- **a.** Add 1/10th (5ul) volume 3M sodium acetate, pH 5.2 (5 μl).
- **b.** Add 2 volumes 100% ethanol (110 μl).
- **c.** Mix by inversion 10×.
- **d.** Incubate at -80 for 30 minutes.
- **e.** Centrifuge at max speed for 30 minutes at 4 °C.
- **f.** Carefully decant off supernatant and blot the tube on paper towels.
- **g.** Add 400 μL 70% ethanol and pipette to re-suspend.
- **h.** Repeat steps e-f 1×.
- **i.** Dry pellets with a vacuum centrifuge for 5-10 minutes, until pellet is dry, or air dry.
- **j.** Resuspend the nick-translated DNA with 100 μL 1× hybridization buffer (see recipe).

Slide pre-hybridization preparation

You will need:

Masked microarray slide(s)

1% BSA Blocking Solution

Graduated cylinder

Coplin Jar

Orbital Shaker (photo 1)

Slide Rinser (photo 2)

Slide Centrifuge (photo 3)

Forceps

De-ionized/distilled water

Kimwipes

Forceps

1. Prepare slides by immersing in 50 ml 1% BSA blocking solution (see recipe) in a Coplin staining jar.
2. Incubate at room temperature for 10 minutes shaking at 80 rpm to eliminate bubbles on the slide surface (photo 1).
3. Dip to rinse slide 20 times in double de-ionized or double distilled water, discard, re-fill water, and repeat for a total of 5 wash cycles (photo 2).
4. Wipe the back and edges of the slide with a Kimwipe and spin dry the slide for 15 seconds using a slide centrifuge. Use forceps to handle the slide (try to only handle slides by frosted end-photo 3).
5. Slides can be stored up to 1 hour before adding the samples, no longer!

Sample Application/Hybridization

You will need:

Nick translated DNA in 1× hybridization buffer

Boiling Water

Container with ice

Masked microarray slides

55 °C waterbath

Pipettes and tips

Humidified chamber (200 μL tip box and lid with de-ionized water covering the bottom of the box)

50 ml conical tube-rack with 50 ml conical tube Styrofoam in the bottom of the rack to hold the ends of the 50 ml conical tubes

Hybridization chamber (50 ml conical tube with filter paper moistened with 1× hybridization buffer)

Lead weights

DO NOT touch the slide surface or let the slide surface dry

1. Boil the nick translated DNA for 3 minutes.
2. Chill on ice.
3. Before using the samples, briefly vortex and centrifuge
4. Place the microarray slide on the humidified chamber. Apply 45 μL of the sample to each well (2 wells/nick translated DNA sample) on the microarray slide. Try to spread the droplet to fully cover the well (photo 4).
5. Carefully seal the slide (face-up and frosted end toward the cap) in a hybridization chamber. The slide will be placed on top of the filter paper in the hybridization chamber. Do not touch the wells with the damp filter paper (photo 5). Be sure the lid is on tight!!
6. Place the hybridization chamber in the rack. Place the lead weight on top of the rack. Submerge the rack in the 55°C waterbath (photo 6). Keep the slides level so samples from other wells do not contaminate each other.
7. Hybridize overnight (12-16 hours).

Post-Hybridization Stringency Washes

You will need:

55°C waterbath

Forceps

Aspirator

Coplin jar filled with pre-warmed (55°C) 1X SSC, 0.2% SDS

1XSSC, 0.2%SDS

1XSSC.

TNT buffer

Horizontal Staining Jar (photo 9)

Orbital Shaker

DO NOT touch the slide surface or let the slide surface dry

1. Pre-warm 1XSSC, 0.2% SDS (recipe) to 55°C in a Coplin Jar.
2. Remove the slides one at a time and immediately aspirate off the excess hybridization solution (photo 7).
3. Completely immerse the slide (frosted end up) in the pre-warmed solution (in the Coplin jar) for 4 minutes (photo 8).
4. Transfer the slide to 0.1XSSC, 0.2% SDS (see recipe).
5. Shake at 80 rpm for 4 minutes at room temperature.
6. Transfer the slide to 0.1XSSC.
7. Gently shake at 80 rpm for 4 minutes at room temperature.
8. Transfer the slide to a horizontal staining jar (photo 9) that contains enough TNT buffer (see recipe) to cover the slide.

9. Shake for 1 minute at 80 rpm at room temperature.
10. Repeat steps 8-9 2 additional times (total of 3 washes).

Development

DO NOT let the slide surfaces dry during the following steps unless instructed to!!

For the following applications, 45 μL of solution is added directly to each well. The slide may be gently tapped to distribute the reagent over the full well surface. DO NOT allow reagents to cross over to other wells.

You will need:

Humidified Chamber (made from a covered tip box with ~10 ml PCR water in the bottom)

Forceps

Perkin-Elmer's TSA kit

TNB

PCR grade water

FES

20X SSC

SA-Alexa 555

50X Denhardt's

TNT buffer

Horizontal Staining Jar (photo 9)

Orbital Shaker

Slide centrifuge

Pipettes and tips

Always prepare the solutions right before use, use 45 μL per well for each solution and incubate at room temperature in a humidified chamber.

1. Spin dry the slides for 5 seconds using a slide centrifuge.
2. Incubate slides with 1:100 SA-HRP in TNB (see recipe E1) for 30 minutes.
3. Wash the slides 3× for 1 minute each in horizontal staining jars at 80rpm shaking.
4. Spin dry the slides for 5 seconds using a slide centrifuge.
5. Incubate slides with 10% FES, 2XSSC (see recipe E2) for 30 minutes.
6. Wash the slides 3× for 1 minute each in horizontal staining jars at 80rpm shaking.
7. Spin dry the slides for 5 seconds using a slide centrifuge.
8. Incubate slides with 1:50 BioT, 1XAmp Dil (see recipe E3) for 10 minutes.
9. Wash the slides 3× for 1 minute each in horizontal staining jars at 80rpm shaking.
10. Dim the lights in the room (leave just enough light to see what you are doing).
11. Spin dry the slides for 5 seconds using a slide centrifuge.

12. Incubate slides with 1:500 SA-Alexa 555, 1XSSC, 5X Den (see recipe E4) for 1 hour **in the dark**.
13. Wash the slides 3× for 1 minute each in horizontal staining jars at 80 rpm shaking.
14. One at a time, wipe the back and edges of the slides with a Kimwipe and spin dry for 15 seconds using the slide centrifuge.
15. Scan the slides. **Store the slides in the dark!**

Scanning/Imaging Slides

1. After hybridization, slides can be scanned or imaged by most standard microarray slide scanners or imagers.
2. Alexa555 has an optimal excitation wavelength of 555 nm and emission wavelength of 565 nm. The system we use (Applied Precision arrayWoRx scanner) has a white light source and an emission filter for Cy3 that functions well for Alex555. We use an excitation wavelength of 540 nm (25 nm bandwidth) and an emission wavelength of 595 nm (50 nm bandwidth). For laser scanners, the wavelength and bandwidth appropriate for Cy3 should be used. We recommend starting with default scanning parameters for Cy3 fluorescence.
3. The array layout is illustrated in the figures below. There are five pairs of Teflon-masked wells on each slide, and the pair of wells furthest away from the frosted end are empty. Each well contains a full array and our normal protocol calls for two wells to be hybridized to the same sample. Within each well there are two spots per probe so in effect there are four individual probe-target hybridizations (2 wells total). Each full array has dimensions of 22 horizontal and 20 vertical spots. The distance between spots is approximately 250 μm.

Reagent/ Supply Appendix

Supplied Reagents

1× Hybridization Buffer

TNB buffer

SA-HRP

FES

Biotinyl tyramide

Amplification diluent

50× Denhardt's

SA-Alexa 555

Materials needed but not supplied

LB broth (Lennox, Cat #BP1427)

Agar (BBL, Cat #299340)

0.22 μm bottle top filter for up to 500 mL volumes (Millipore, Cat. #SCGPT05RE)

0.22 μm bottle top filter for 0-200 mL volumes (Millipore, Cat. #SCGPU01RE)

0.45 μm sterile syringe filter (Fisher Scientific, Fisherbrand Cat. #09-179D)

Double de-ionized water

NaCl (Sigma-Aldrich Cat. #S1679)

Trisodium Citrate Dihydrate (JT Baker 3646)

HCl (JT Baker 9535)

BSA (Fisher BP1600)

SDS (Serva, Cat. # 39575)

Tris-HCl (Sigma-Aldrich Cat. #93363)

Tween-20 (Polyoxyethylenesorbitan Monolaurate – Sigma-Aldrich Cat. #P2287)

Reagent Recipes

LB broth 20g LB broth mix
DD water to 1L
Autoclave and aliquot to 3ml tubes

LB agar: 20g LB broth mix
15g agar
DD water to 1L
Autoclave and pour plates aseptically.

20X SSC

1. Using a 500 mL beaker with a stir bar, dissolve the following (while stirring) in ~450 mL nanopure de-ionized water:
    a. 87.7 g NaCl
    b. 44.1 g Trisodium Citrate Dihydrate
2. Adjust the pH to 7.0 with ~2 drops of concentrated HCl
3. Transfer to a 500 mL volumetric flask and bring the volume up to 500 mL with nanopure de-ionized water.
4. Transfer to a 500 mL bottle and autoclave.

1% BSA Blocking Solution

1. Combine the following in 100mL nanopure de-ionized water:
    a. 5 g BSA
    b. 75 mL 20X SSC
2. Add nanopure de-ionized water to a final volume of 500 mL.
3. Filter sterilize.

SSC Stringent Array Washes

High Stringency: 0.1X SSC

1. Make 0.1X SSC by placing 2.5mL 20X SSC in a 500mL graduated cylinder and adjusting the volume with nanopure de-ionized water to a final volume of 500 mL.
2. Filter sterilize by using a bottle top filter and pour into a 500 mL bottle. (Save the filter for the next two reagents).

Medium Stringency: 0.1X SSC, 0.2 % SDS

1. Combine the following with ~250 mL nanopure de-ionized water:
    a. 2.5 mL 20X SSC
    b. 5 mL 20% SDS
2. Add additional nanopure de-ionized water for a final volume of 500 mL
3. Filter sterilize by using the bottle top filter from high stringency filtration and pour into a 500 mL bottle.

Low Stringency: 1X SSC, 0.2% SDS

1. Combine the following with ~250 mL nanopure de-ionized water:
    a. 25 mL 20X SSC
    b. 5 mL 20% SDS
2. Add additional nanopure de-ionized water for a final volume of 500 mL
3. Filter sterilize by using the bottle top filter from previous filtrations and pour into a 500 mL bottle.
4. Do not autoclave.

TNT Buffer

For a final volume of 1L:

1. Measure 850 mL de-ionized nanopure water in a graduated cylinder or volumetric flask.
2. Add the following reagents:
    a. 100 mL 1 M Tris-HCl, pH 7.5
    b. 30 mL 5 M NaCl
    c. 500 μL Tween-20
3. Bring volume to 1L using de-ionized nanopure water, seal cylinder with parafilm or cap flask – mix by inverting several times.
4. Filter sterilize using a bottle top 0.2 μm filter into a bottle.

TSA Detection Recipes

(make 100 μL per sample (two wells) and use 45μL aliquots per well)

E1. For 1:100 SA-HRP, TNB: add 1μL SA-HRP to 99μL TNB

E2. For 10% fetal equine serum (FES) in 2X SSC: add 10 μL FES and 10 μL 20X SSC to 80 μL PCR water

E3. For 1:50 biotinyl tyramide (BioT), 1X Amplification Diluent (AmpDil): add 2 μL BioT (thaw before using) to 98 μL AmpDil

E4. For 1:500 SA-Alexa 555, 1X SSC, 5X Den: add 0.8 μL SA-Alexa, 20.0 μL 20X SSC, and 40 μL Den to 339.2 μL PCR water

photo 1.
BSA Immersion

photo 2.
Slide Rinse

photo 3.
Slide Spin

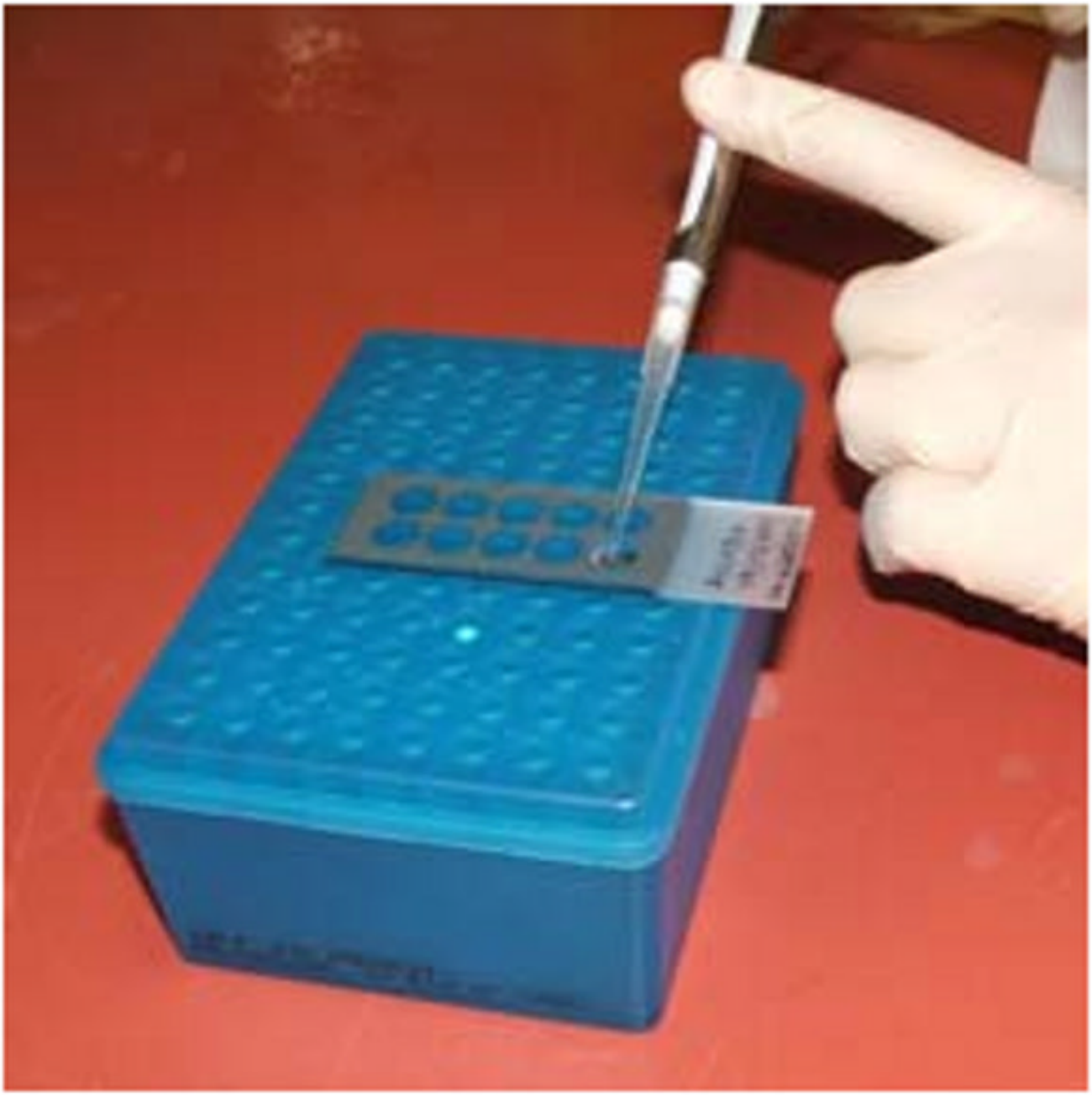

photo 4.
Sample Application

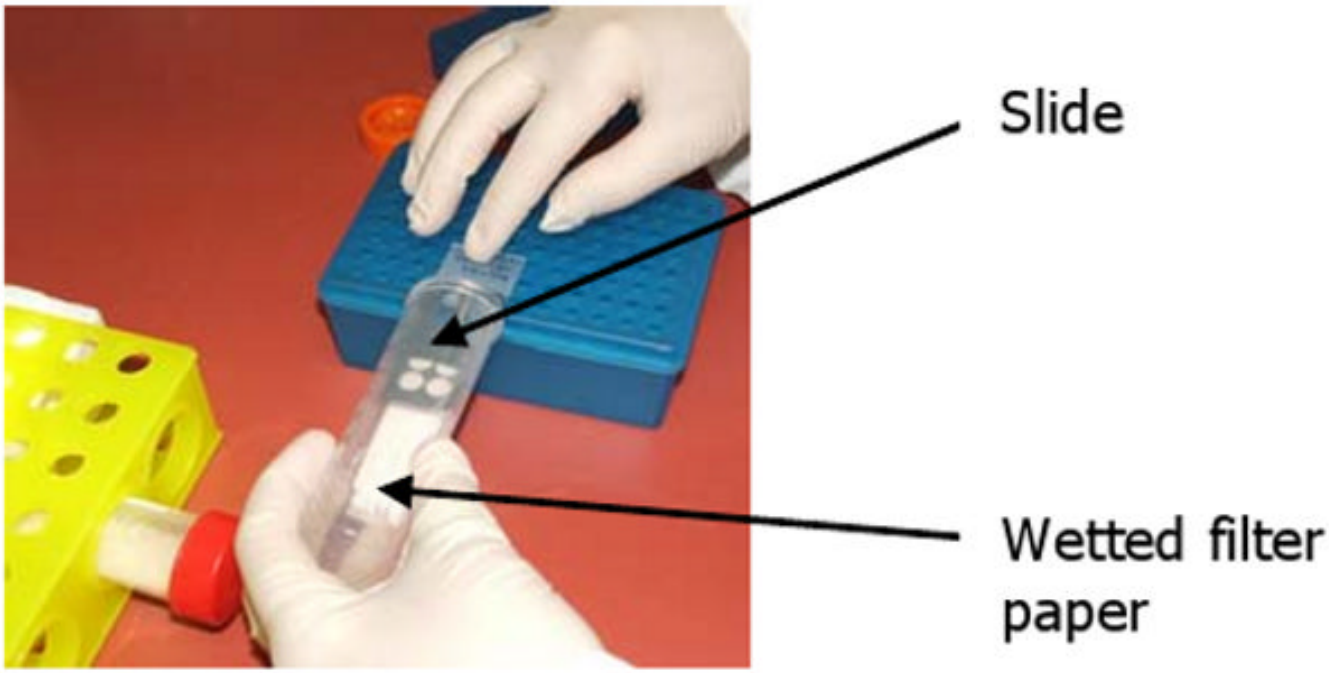

photo 5.
Hybridization Chamber

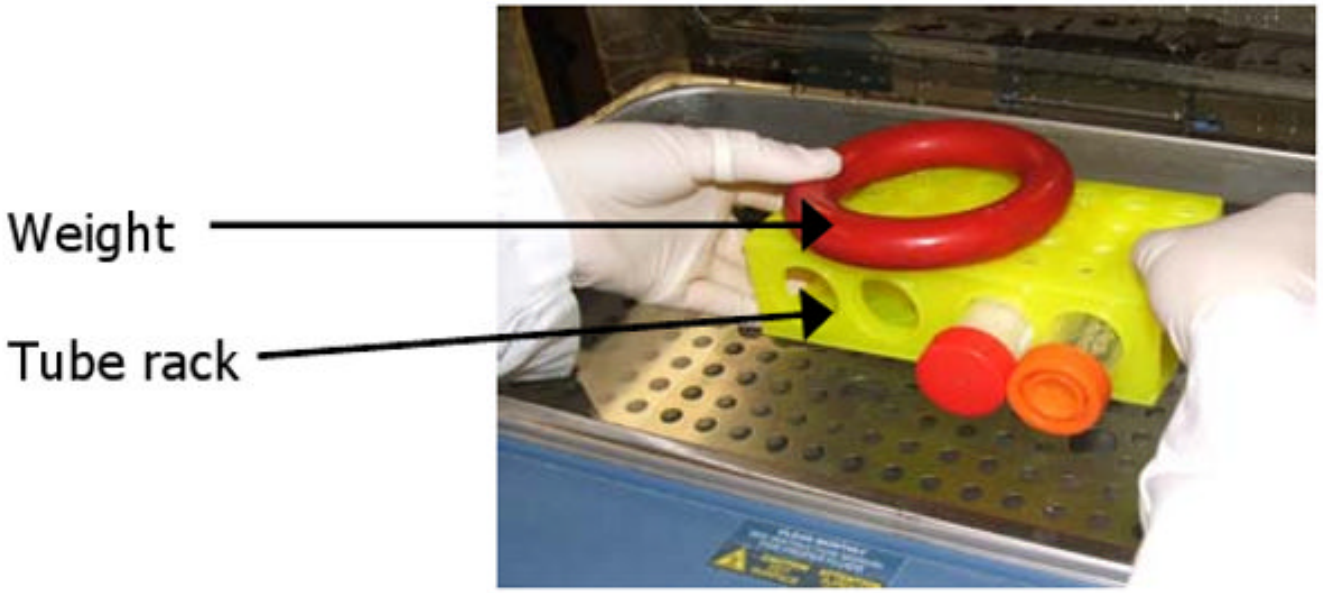

photo 6.
Immersion in 55 °C Water Bath

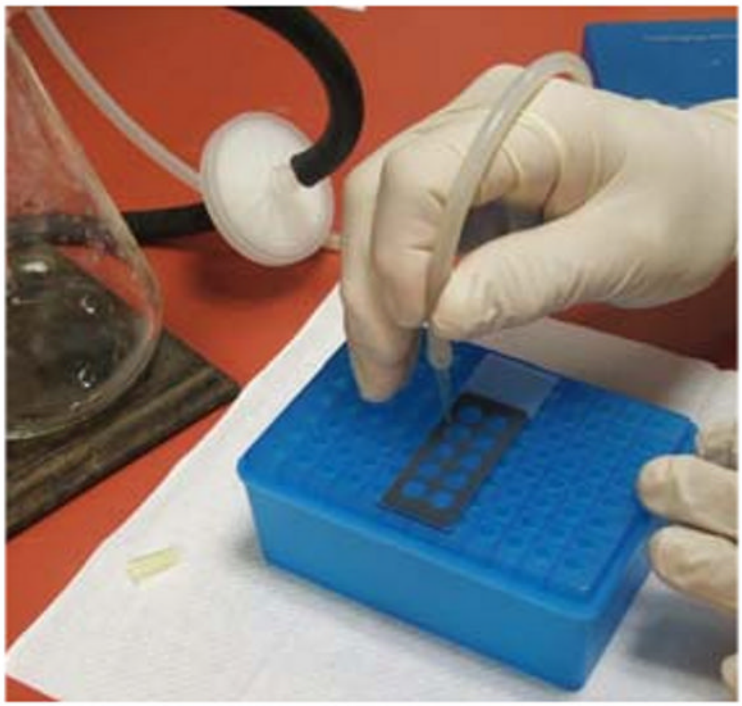

photo 7.
Slide Aspiration on Chamber/Tip Box

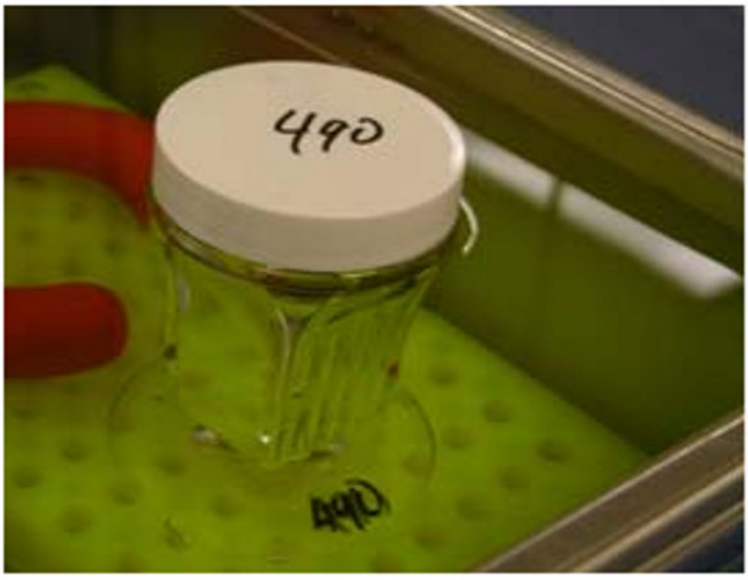

photo 8.
Coplin Jar

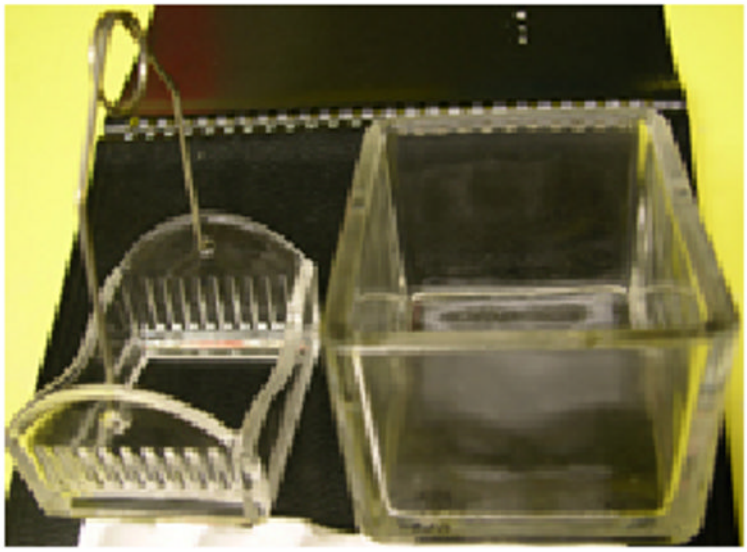

photo 9.
Horizontal Staining Jar

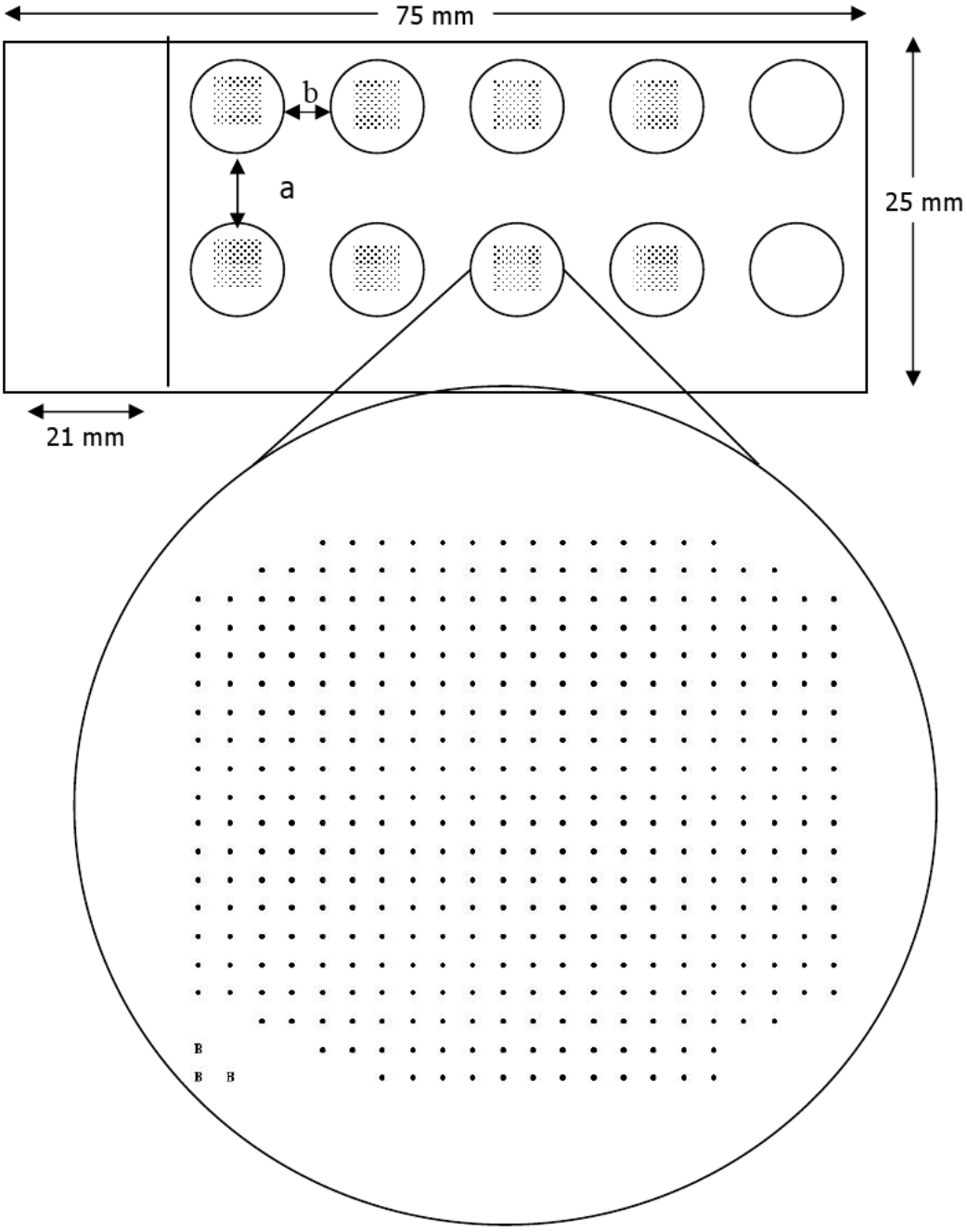

Figure 1. The two wells furthest from the frosted end of the slide do not contain printed arrays
The letters "B" indicate the location of the biotin positive control spots. Well diameter ~ 7.9mm, between wells a) ~1.1 mm, b) ~0.95 mm.

	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22
1			TetB	TetG	TEM1	aadA2	floR	qac	strA		
2		strB	bla carb-2	Sul1	Sul2	TetE	OtrB	TetH	TEtO	TetS	
3	TetW	TetM	Tet30	tetC	tetY	tetD	TetU	TetK	TetQ	TetX	TetT
4	TetU	TetV	Aph(3')-Ia	aph(3')-IIa	aph4	aphA-3	aphD	aphE	aphIII	bla-CMY-2	blaCTX-M-1
5	bla-CTX-M-12	bla-DHA-1	bla-FOX-2	blaIMP-2	blaKPC-3	bla-OXA-1	bla-OXA-2	bla-OXA-27	bla-OXA-2b	bla-OXA-6	bla-OXA-7
6	bla-OXA-9	bla-OXY-K1	bla-PER-2	bla-PSE-1	bla-PSE-4	bla-ROB-1	bla-SHV-37	catI	catII	catIII	cblA
7	dhfrII	dhfrIII	dhfrIX	dhfrIV	dhfrVI	dhfrVIII	dhfrXII	dhfrXIII	dhfrXV	aidaI	aafA
8	aap	aatA	aggA	pet	ehxA	stx1A	stx1B	tir-2	invX	ipaB	sopA
9	bfpA	eaf	espC	cif	eae	fotA	leoA	stII	fl65(1)A	sfaA	sfaD
10	sfaHII	saa	hlyA	papG12	usp	wzya(1-2)	rfbE(A_D)	abe(C2-C3)	wbaV(B)	wbaU(B_D)	wbaA
11	wbaV(D1)	wbaO(E1_D2)	TetA	aadA1	bla-CTX-M-2	bla-CTX-M-8	catP	dfrA1	aac(3)-Ia	aac(3)-Ib	aac(3)-Id
12	aac(3)-III	aac(3)-IVa	aac(3)-Vb	aac(6')-Ia	aac(6')-Ib	aacC1	aacC2	aadA7	aadB	aadE	bla-ACC-01
13	bla-MIR	cat	cat4	cmlA	cmlB	Dfr1	dhfrI	dhfrVII	dhfrX	IntI1	IntI2
14	DT104	SSpp	sefA	164	sipC	IncP / trfA2	IncN / kikA	IncW / trwAB	IncFII / Ori	Lt	stb
15	fliCH7	rfbE	estA	spvR	spvC	sipA	invA	fliC	pagC	mpha	ent
16	parAB	Iterons H12	RNAI I1	Ori gamma X	pir X	repA L/M	repAM L/M	repC L/M	repA N	Iterons FIA	repA FIB
17	repA W	repA Y	Iterons P	repA2 FIC	repA FIC	repA T	RepA FIIS	RNAI/repA	RNAI K/B	RNAI B/O	catB2
18		catB3	catB8	aadA5	aadA21	aacCA5	aac(6')-I30	aac(6')-Iia	aphA7	dfrA1	
19	Biotin		dfrA16	dfrA19	dfrA2	dfrA23	sul3	LTIIa	qnrA1		
20	Biotin	Biotin		qnrB	qnrS	Stx2A	Stx2B	Bla-CTX-M-14	TetQ		

Figure 2.
Locations of gene probes on array.

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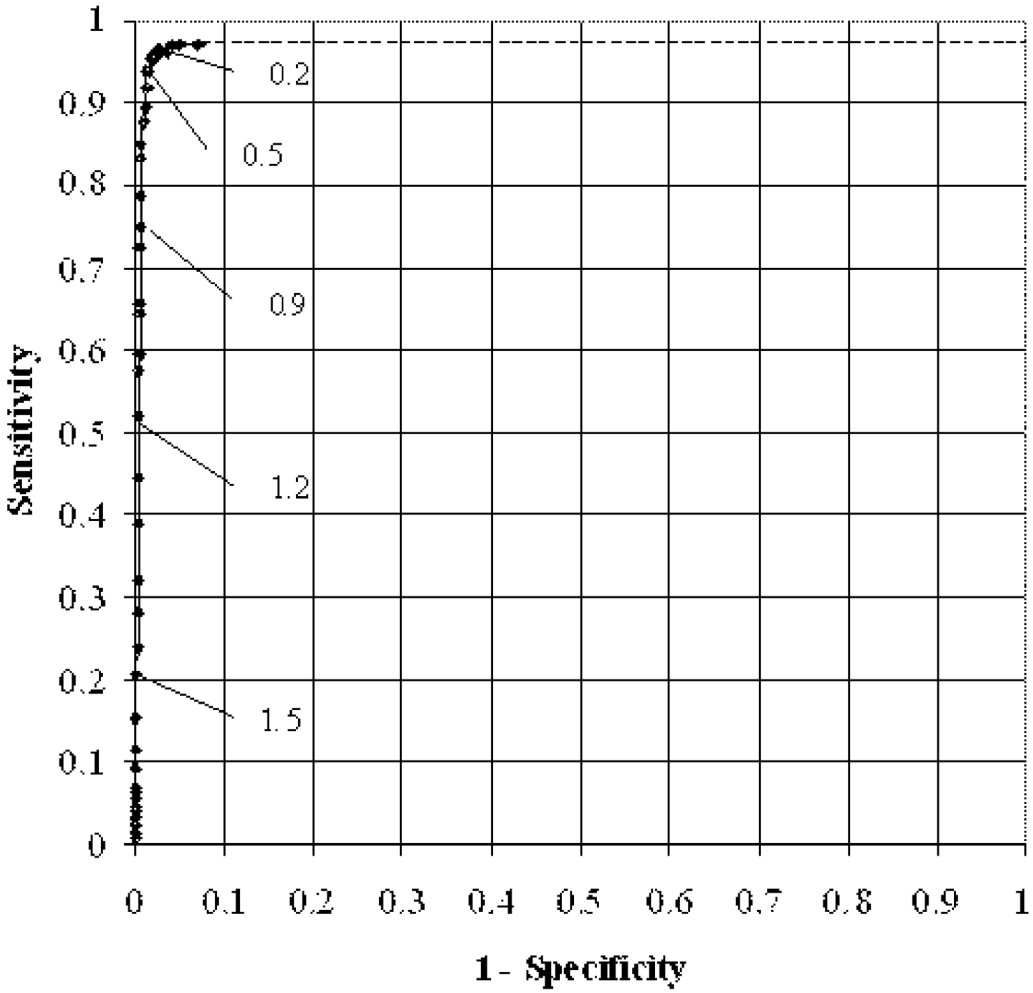

Figure 1.

Receiver-operator characteristic (ROC) curve using sensitivity and specificity values based on thresholds for a positive result at increments of 0.05 normalized fluorescence intensity. The area under the curve = 0.97. Numbers inside the graph indicate thresholds associated with data points.

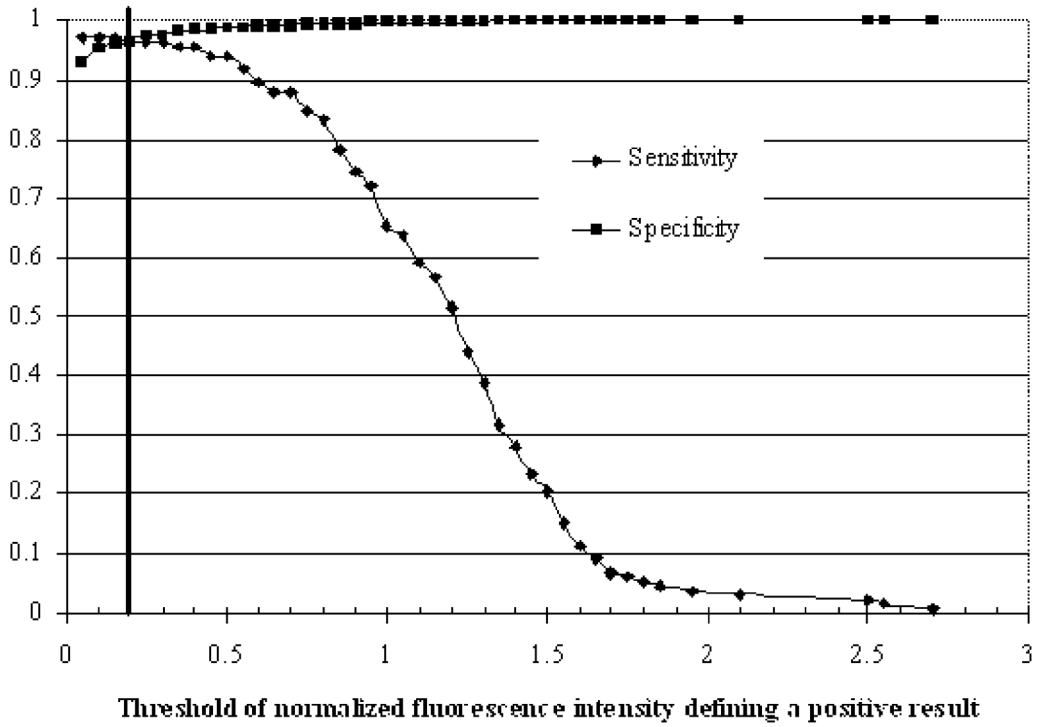

Figure 2.
Sensitivity and specificity for normalized fluorescence intensity threshold values at increments of 0.05.

Table 1

Bacterial isolates and gene probes used to generate the ROC curve and used in the blinded validation study.

Description		Isolate (Reference)	Genes for which this isolate is a positive control
Sequenced *E. coli* O157:H7	1.[a]	EDL933[b]	*eae, ehxA, fliCH, repA FIB, rfbE, RNA1/repA, stx1A, stx1B, sStx2A, stx2B, tir-2*
Sequenced *Salmonella* Typhimurium	2.	LT2[c]	*ent, fliC, invA, pagC, repA FIIS, sipA, sipB, sipC, spvC, spvR, SSpp, wbaU (B_D), wbaV(B), wzya(1-2)*
Typhimurium DT104 ACSSuT [d]	3. [a]	S2057[e]	*aadA2, blaPSE1, floR, qac delta E, sul, tetG, wbaU (B_D), wbaV (B),*
Typhimurium AKSTCaz [d]	4. [a]	S8200[f]	$bla_{CMY-2,}$ *sipA, sipB, sipC, spvC, spvR, SSpp, wbaU(B_D), wbaV(B), Wzya(1-2)*
Newport ACGKSxtST [b]	5.	S6615[g]	*abe(C2-C3*)
Newport ACSTAmcCaz[b]	6.	S10869[h]	*abe(C2-C3*)
E1 Uganda ACGKSxtSTAmcCaz [b]	7.	S11272[i]	*wbaO(E1_D2*
Genes in *E. coli* background	8.	OtrB clone[j]	*otr*B
	9.	Q116 [k]	bla_{SHV}
	10.	Tet30 clone[j]	*tet30*
	11. [a]	TetA clone[j]	*tetA*
	12.	TetB clone[j]	*tetB*
	13.[a]	TetD clone[j]	*tet*D
	14.	TetH clone[j]	*tetH*
	15.	TetO clone[j]	*tetO*

[a] Isolates that were also used for the blinded validation study

[b] (Perna, et al., 2001); (Burland et al., 1998).

[c] (McClelland, et al., 2001).

[d] A, ampicillin; C, chloramphenicol; K, kanamycin; S, streptomycin; Su, Triple-sulfa; T, tetracycline; Caz, ceftazime.

[e] Isolated from bovine feces in 1990 and determined to be phage type DT104 by phage typing.

[f] (Kang et al., 2006).

[g] (Kang, et al., 2006).

[h] CDC isolate AM04528.

[i] Isolated from bovine hide swab in 1996.

[j] (Call, et al., 2003).

[k] (Randegger and Hachler, 2001)

Table 2

The number of gene probes on the array according to their class of antibiotic resistance or description.

Gene description	Number of probes on the array
Aminoglycoside resistance	30
Beta-lactam resistance	27
Disinfectant resistance	1
Erythromycin resistance	1
Phenicol resistance	12
Quinolone resistance	3
Sulfonamide resistance	3
Tetracycline resistance	21
Trimethoprim resistance	19
DT104 marker	1
E coli pathotype	29
Integrase gene	3
Misc. recombination hotspot	1
Replicon type markers	25
Salmonella-specific markers	3
Salmonella serogroup markers	8
Virulence genes	16
Total	203

Table 3

Kappa statistics using a 0.2 threshold.

	Data from all probes and isolates (n = 1,015)	Data from all probes, excluding isolate 36 (n = 812)	Data from probe/isolate combinations with known positive and negative values (n = 225)
Laboratory pair	Kappa (95% CI[a])	Kappa (95% CI)	Kappa (95% CI)
AB	0.46 (0.31, 0.61)	0.47 (0.32, 0.620	0.40 (0.12, 0.68)
AC	0.28 (0.20, 0.36)	0.34 (0.25, 0.43)	0.13 (0.03, 0.24)
AD	0.17 (0.07, 0.27)	0.21 (0.09, 0.32)	0.11 (-0.06, 0.27)
BC	0.33 (0.25, 0.41)	0.38 (0.29, 0.47)	0.35 (0.21, 0.49)
BD	0.14 (0.04, 0.23)	0.13 (0.03, 0.24)	0.20 (0.01, 0.39)
CD	0.31 (0.23, 0.40)	0.22 (0.13, 0.31)	0.52 (0.38, 0.66)
ICC[b] (95% CI)	0.27 (0.24, 0.29)	0.28 (0.25, 0.31)	0.29 (0.23, 0.34)

[a] CI, confidence interval

[b] ICC, intraclass correlation coefficient