Agilent SurePrint Technology

Content centered microarray design enabling speed and flexibility

Agilent Technologies

SurePrint: the concerted effort of mechanical, biological, and chemical parameters working synergistically to create the highest quality microarrays.

SurePrint is more than just the best technology or use of the highest quality reagents. It is the culmination of technological ingenuity, continual quality control monitoring, and human intervention throughout microarray creation ensuring that everything is working at the peak of performance. This unique combination of elements enables Agilent to guarantee that researchers receive only the highest quality catalog and custom microarrays.

Workflow

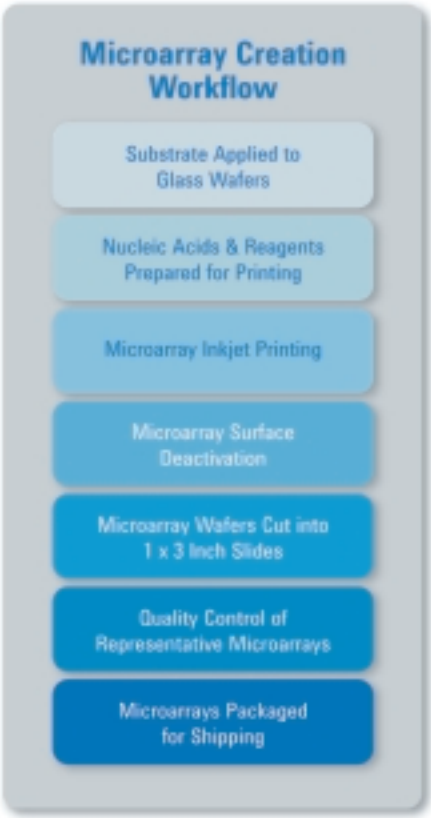


Figure 1.

There are seven main steps in the creation of Agilent microarrays (Figure 1). First, glass wafers are coated with a surface that will make a strong bond with both the glass and the nucleic acids that are to be printed. Then, the reagents for oligo synthesis are inspected for quality and purity, while the cDNA for deposition microarrays is prepared and rigorously qualified for printing. Next, the prepared glass and the nucleic acids come together through the careful orchestration of inkjet printing, which involves multiple real-time quality control feedback mechanisms to monitor the presence, size, shape and position of every feature. Following printing, the microarrays undergo a process to permanently bind the printed DNA to the surface of the microarray, and deactivate the surface around the features. This minimizes the surface's ability to bind non-discriminantly with sample that could lead to high background signal.

At this stage, microarrays printed on large sheets of glass wafers are cut into individual one by three inch slides and are bar-coded for identification. The microarrays are set aside while microarrays from each print run are inspected by quality control. The fate of the entire print run is dependent upon the performance of the microarrays in the QC evaluation. They are hybridized with both control and reference targets for rigorous analysis to ensure that a minimum of 95% of the experimental features are present, hybridizable, extractable, and uniformly printed. Microarray batches that pass the stringent quality control inspections are then packaged and prepared for shipping. At each stage in the workflow, system checks allow only high quality products to progress on to the next step. Intermediates that don't pass the intermediate QC checks do not proceed forward, ensuring that the final microarrays shipped to our customers are of superior quality.

A Closer Look At The SurePrint Process

Glass Inspection & Surface Application

The final product is only as good as the elements used to make it. Agilent pays close attention to the quality of glass used for microarrays, because poor quality glass limits scan quality even with a state-of-the-art microarray scanner such as the Agilent DNA microarray scanner with SureScan technology (Figure 2). Glass warps easily during production and typically shows variations in thickness and texture, creating a significant challenge to many commercial scanners attempting to measure microarray features on a variable surface. The Agilent scanner measures pixel intensity in the horizontal plane of the slide where the features are printed, but it also compensates for surface roughness, curvature and warp of the slide in the vertical dimension. Other scanners remain sensitive to these sources of noise. To optimize conditions for scanning, the glass must be a uniform thickness, have a smooth surface free of aberrations, free of curvature or warping, exhibiting minimal inherent fluorescence. Agilent purchases only high quality glass that meets all of these specifications.

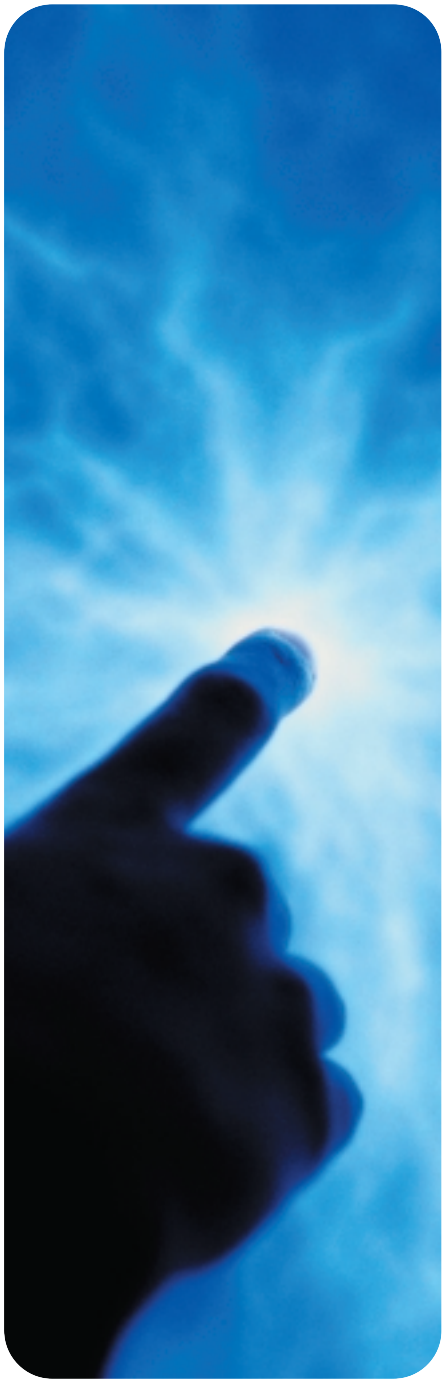
Nucleic acids do not inherently bind to glass. Application of a thin film of an activated monolayer substrate makes the glass surface of the microarray conducive to spotting and acts as an anchor to secure the printed nucleic acids tightly to the surface. The substrate monolayer must be uniformly applied, resistant to delamination, and encourage consistent spot morphology and placement. Substrate delamination is tested by submersion into the hybridization solutions to check for any lifting or peeling away from the glass surface. Next, control solutions are printed onto the substrate and are evaluated by their drop characteristics through a measurement of the spot contact angle to ensure that the surface produces uniform spots. Then, a visible inspection of the spot placement and morphology is done to evaluate whether the spotted features interact with the glass to form orderly rows and columns. The glass is rejected if the substrate does not interact well with the control solutions.

Figure 2. This image shows how warped or curved slides can lead to out of focus images when an initial single point of focus is used to focus the optics on the microarray surface. Compensation in the vertical dimension is not a problem for the Agilent DNA microarray scanner with autofocus, so that all features on the microarrays surface are in perfect focus.

Probe Selection

Agilent creates two types of microarrays: 1) deposition microarrays in which cDNA or presynthesized oligonucleotides are prepared off line and then deposited onto the surface of the microarray, and 2) *in situ* synthesized oligonucleotide microarrays in which the oligos are synthesized directly on the glass microarray surface. Agilent offers catalog microarrays in both the cDNA and oligo formats so that researchers have the best choice of products for their experimental needs. Content is selected to provide the highest quality content on the microarray for the given microarray format. To learn more about how Agilent microarray content is selected and qualified, please see the technical paper "The Bioinformatics Behind Microarray Creation."

In contrast to catalog microarrays, the sequences of the custom oligos are designed around the specific experimental goals of the researcher through a consultative working relationship with Agilent's microarray design experts. As part of the custom microarray design service, the best set of probes are designed based on such criteria as the T_m, base composition, orientation, basic probe suitability, and low potential for cross-hybridization. The primary microarray design is then printed and delivered to the customer. Through an iterative process, the oligos can be optimized experimentally to create very targeted, highly functional, custom microarrays.



Reagent Preparation

Based on standard phosphoramadite chemistry, oligonucleotides on our *in situ* microarrays are synthesized base by base to create oligos for both custom and catalog microarrays. Thousands of oligos are simultaneously synthesized on a solid glass surface, one layer at a time, using the building blocks for DNA molecules. After the oligo synthesis reagents are qualified, high nucleotide coupling efficiency results in successful long oligo synthesis for microarrays with extremely high sensitivity.

In contrast to synthesized oligo microarrays, deposition microarrays are printed on the microarray surface using DNA molecules prepared prior to printing. Agilent's cDNA microarrays are printed with clone PCR products from Incyte Genomics' LifeSeq clone sets. All of the PCR clones are sequence-verified by Incyte to ensure that they match up correctly with their identity in the database. They are purified and run on gels, checking for clone size and the absence of multiple bands. This reconfirms the identity of the PCR products and ensures that there is no cross contamination. Then, the DNA concentration is measured; clones not meeting minimal DNA concentrations to achieve optimal gene expression performance are flagged. Incyte guarantees the quality and purity of the clone content it provides Agilent, so researchers can be assured of optimal probe performance for each clone printed on the microarrays.

Inkjet Printing with Real Time Visual Monitoring

The idea behind printed microarrays emerged from Agilent's long printing legacy as part of Hewlett Packard. HP's innovative printing technology makes it a market leader in the printing world. Similarly, Agilent is a formidable provider of consistent, affordable, high quality, printed microarrays through the speed, flexibility and volume that inkjet printing enables in microarray production.

Printing microarrays is similar to printing a document with an inkjet printer. The printer consists of an inkjet head with nozzles through which ink can flow onto the paper. The nozzles are controlled by a computer which directs where to deposit ink, what color ink to use, and how much ink to deposit. The printer also contains cartridges that act as reservoirs holding a supply of ink to print with. Similar components make up an Agilent microarray printer which prints nucleic acids onto an activated glass surface instead of paper.

Washing the inkjet head is the first step in printing microarrays. It removes any contaminants that may clog the nozzles or cause poor microarray performance. DNA samples are then loaded and test fired. The test fire determines which nozzles produce uniform spots with the most accurate spot placement. Only the best nozzles will be used to print the microarrays. Once the nozzles are selected, the cDNA samples are printed onto the microarrays.

The printer spots very precise, small volumes of liquids; only a few microliters of cDNA are necessary to print thousands of microarrays. The first set of clones is sequentially printed via non-contact ink-jet deposition on every microarray, of each wafer in a batch. Each time a nucleic acid is printed, the spot, alternatively called a feature, is visually inspected in real-time to verify its presence, size, shape and location. Real-time monitoring ensures that the entire batch is consistent from the first feature to the last.

After the first set of cDNA clones is printed, the reservoirs are emptied and rinsed. Then the inkjet head is washed and flushed thoroughly so that there is no cross contamination with the next round of cDNA clones to be printed. This process of loading cDNA clones, printing and washing is repeated until all control and experimental features have been printed on each microarray. Once all of the features have been printed, the microarray wafers go through a step to deactivate the surface substrate that cDNA was not printed on. This lowers background fluorescence by preventing non-specific binding to the microarray surface.

The process of printing oligonucleotide microarrays is nearly identical to the process described for cDNA microarrays, however, instead of printing the oligos fully prepared onto the microarray surface, they are actually synthesized base-by-base in repetitive print layers using standard phosphoramidite chemistry (Figure 3). After the inkjet head and reservoirs are washed and thoroughly dried, they are connected to bottles containing the four different phosphoramidite nucleotides that make up the building blocks of *in situ* nucleic acid synthesis. This ensures a constant supply of reagents flowing to the inkjet head during printing.

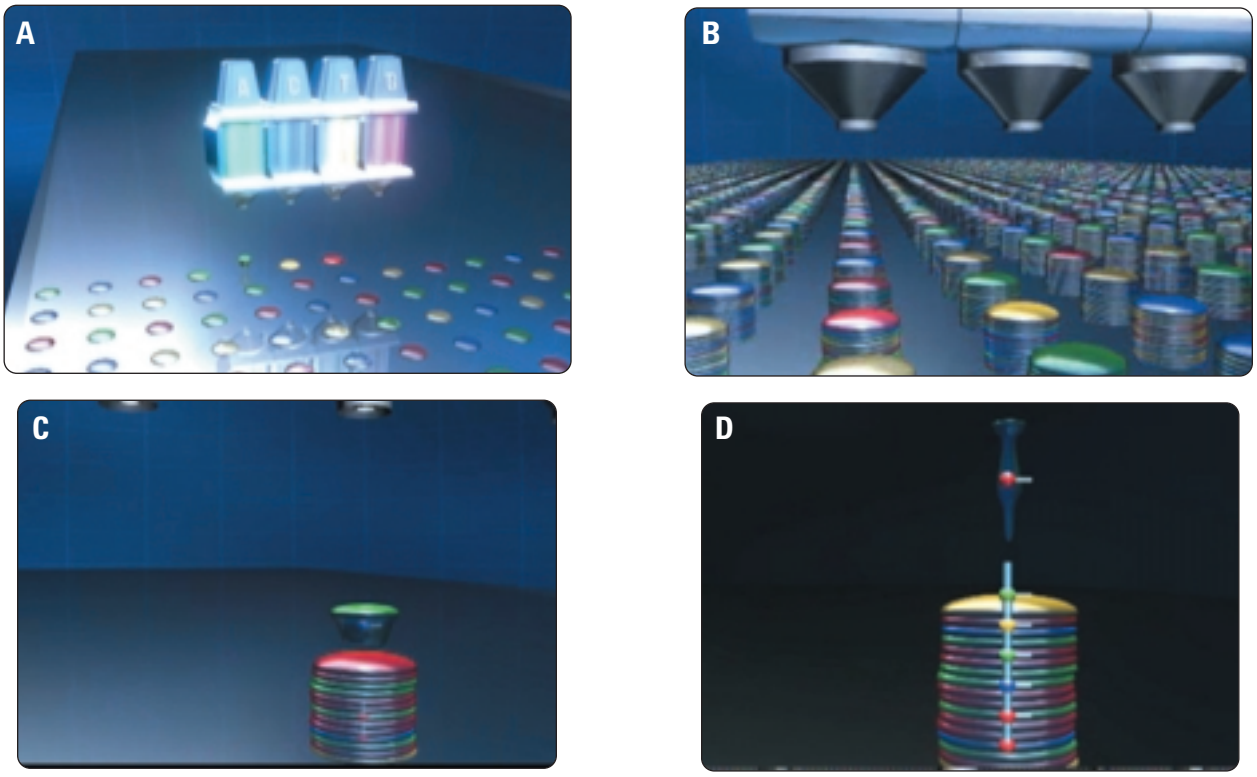


Figure 3. These four images communicate the general mechanism for oligo synthesis via inkjet printing. **A** shows the first layer of nucleotides being deposited on the activated microarray surface. **B** shows the growth of the oligos after multiple layers of nucleotides have been precisely printed. **C** is a close-up of one oligo as a new base is being added to the chain, which is shown in figure **D**.

The oligo print run commences with the firing of a test pattern to select the best nozzles for printing. Then the iterative oligonucleotide synthesis loop begins when the first nucleotide of each oligo is printed onto the activated glass surface of the microarrays. In phosphoramidite synthesis reactions, the reactive sites on the nucleotides are blocked with chemical groups that can be removed selectively. This allows the bases to be added to the oligo chain one base at a time in a very controlled manner. After the first base is printed, the trityl group that protects the 5′ hydroxyl group on the nucleotide is removed and oxidized to activate it, enabling it to react with the 3′ group on the next nucleotide. In between each step, the excess reagents are washed away so that they won't randomly react later in the synthesis. This process is shown in Figure 4. The process of printing a nucleotide followed by de-tritylation, oxidation and washing is repeated 60 times. After the last base in the oligo chain is printed, the microarrays undergo a final deprotection step, before moving on to scribing, dicing and quality control testing.

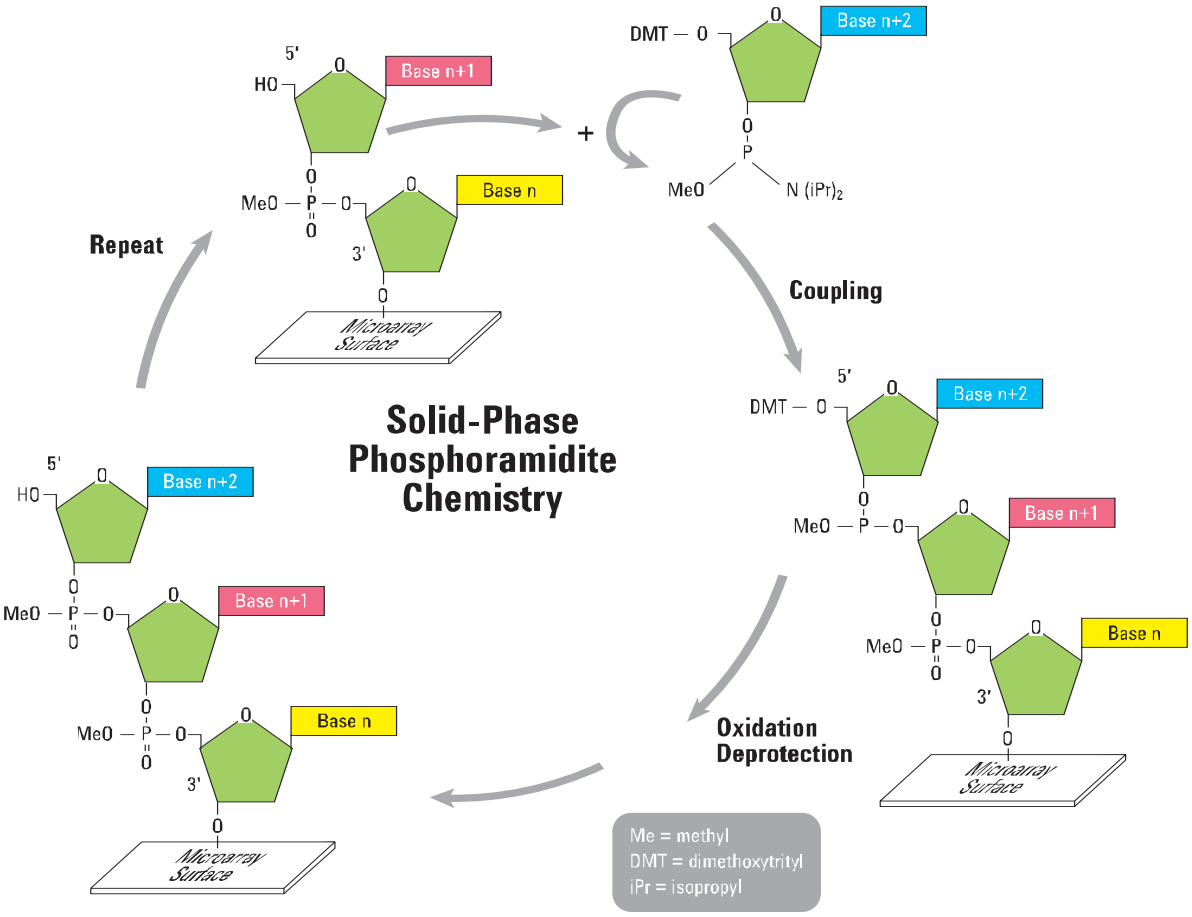


Figure 4. This figure shows the general cycle of oligo synthesis via phosphoramidite chemistry.

Scribing and Dicing

Glass is a difficult material to cut because it is hard, amorphous, exhibits resistance to shearing stresses, and cracks easily. The type of glass being cut will largely determine the technique and blade specifications used to cut it. Agilent uses two main techniques to cut microarray wafers into standard one by three inch slides. The cDNA microarrays are cut using a Disco High-Tech American Saw, which is commonly used in the semi-conductor industry to dice silicone wafers. The Disco Saw is programmed to complete complex processes such as wafer alignment and contoured edge profiles, and is an efficient way to precisely cut large volumes of glass.

In contrast, the oligonucleotide microarray wafers are separated into individual microarrays in a two-step process. First, fine lines are etched into the surface of the wafer using a CO_2 laser scriber. The laser heats up the glass and produces fine scribe lines. A fog of ethanol is applied to the surface following the laser scribing to quickly cool down the glass surface. The wafer is then easily broken along the scribelines using a CBS "Crop, Break, and Snap" tool.

The edge quality of the individual microarrays from both cutting processes is scrutinized for roughness along the new edges that may interfere with slide hybridization and scanning. The slides are then inspected for proper alignment; the active microarray surface must be precisely centered on the slide. Barcodes are applied to the newly sliced and diced microarrays, which are then checked into the database. Select microarrays from the beginning, middle and end of each print run are sequestered for rigorous quality control testing, while the rest of the print run is set aside. If the representative microarrays do not pass quality control, none of the microarrays from that print run will be packaged and shipped to customers.

Quality Control

Agilent's QC process is very robust. Not only are QC tests incorporated into every step in the manufacturing process, microarrays then undergo rigorous performance testing at the end of the workflow to confirm successful performance with actual samples before microarrays in the batch are sent to customers. Representative QC microarrays are pulled from every wafer in each batch for testing. All of the QC information is stored in a database, which can then be linked back to each individual microarray through its barcode. Agilent guarantees that each microarray contains a minimum of 95% of the features are on the microarray. However, in addition to being present, the location, feature uniformity, sensitivity, reproducibility and hybridization performance are also guaranteed.

The QC tests hinge on being able to test every feature printed on the microarray. For deposition microarrays (Figure 5) this is simple because all of the exact cDNA sequences are known and consistently spotted onto a large number of microarrays. A sample target specifically designed to successfully hybridize to every probe on the catalog cDNA microarrays is utilized in the testing. Custom oligonucleotide microarrays are tested differently because only a limited number of microarrays will be printed with the custom sequences. It is impractical to generate a reference sample for each probe on custom microarrays. However, this does not mean that the custom microarrays cannot be measured to the same quality standards as the catalog microarrays. Instead, special "QC" oligonucleotide microarrays, with a standard set of oligos, are incorporated into each oligonucleotide microarray print run (Figure 6). A fluorescently labeled reference sample complementary to every oligo on the QC microarray is then used in performance tests to guarantee the quality and performance of the microarrays. The QC microarrays are printed at specific locations throughout the batch to ensure that oligo synthesis is the same at the beginning, middle and end of the batch. The oligo synthesis on the QC microarrays is identical to the oligo synthesis on the catalog and custom oligo microarrays because the QC oligos are simultaneously synthesized with the custom oligos in an iterative process, one layer at a time. If the oligos on the QC microarrays perform within the specifications, the custom oligonucleotides will also perform according to specifications.

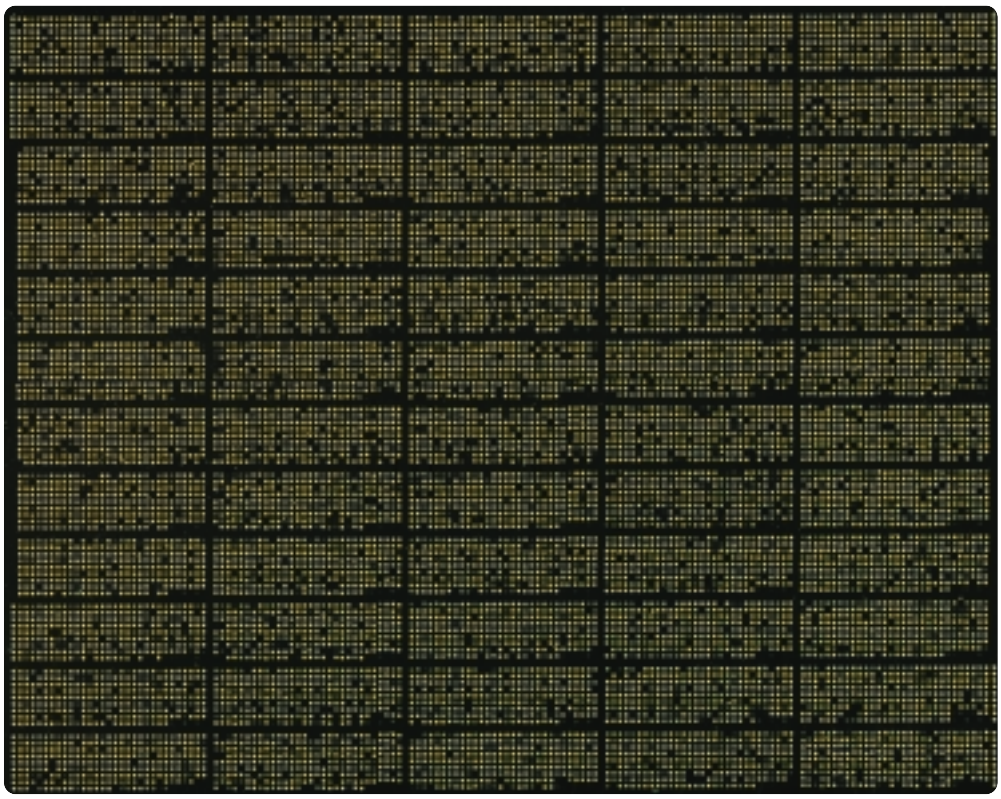


Figure 5. Almost all of the features on the cDNA microarray light up when hybridized with a target sample complimentary to every cDNA deposited on the microarray.

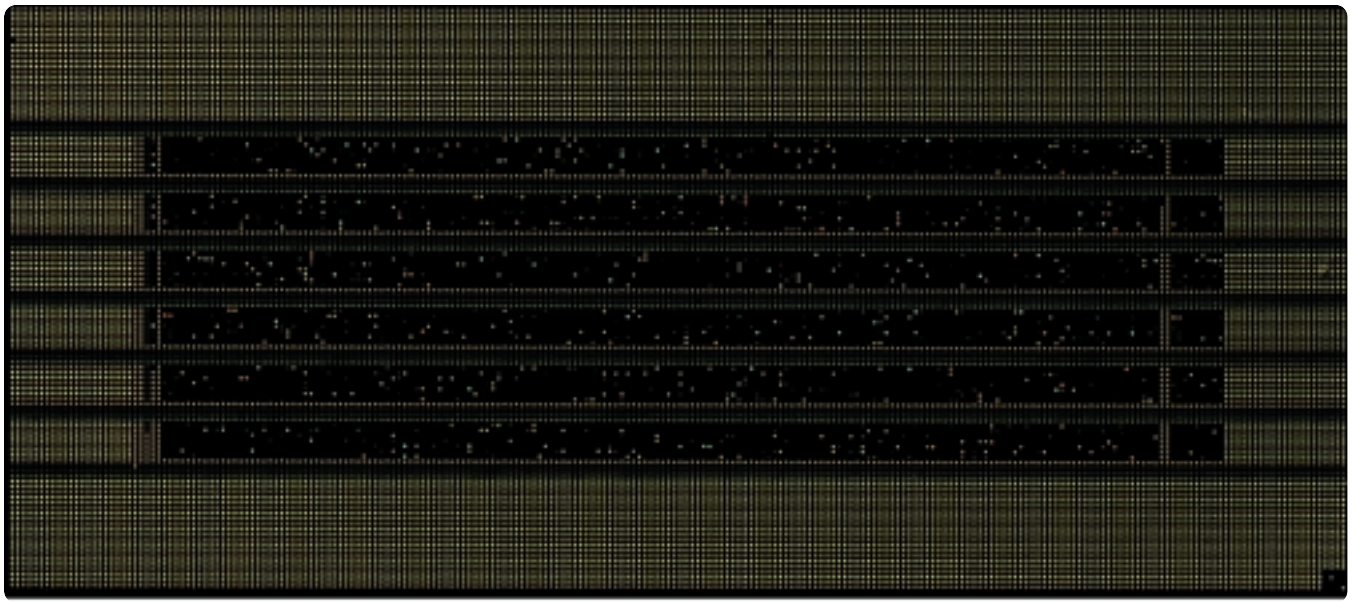


Figure 6. This is a hybridized image of a QC oligonucleotide microarray that is printed at multiple positions on the wafer any given print run along side both custom and catalog oligonucleotide microarrays.

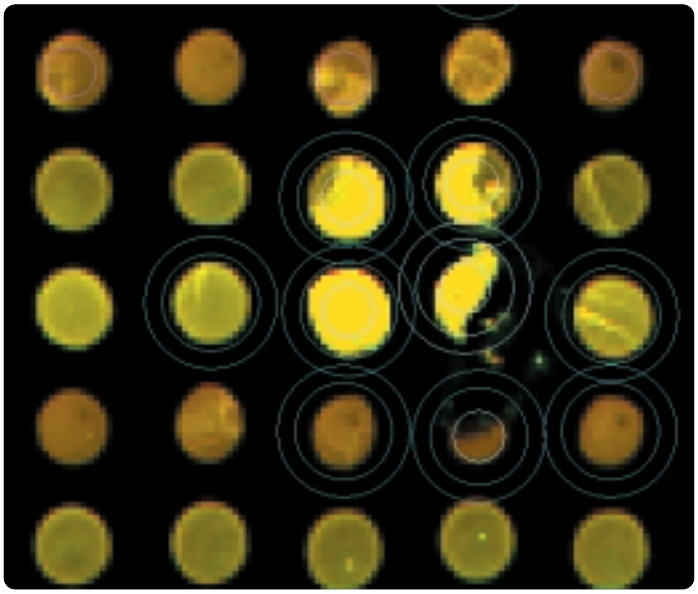


Figure 7. Features identified by the non-uniformity flagger in Agilent's Feature Extraction software appear with blue and pink circles to emphasize features with poor spot morphology or uniformity.

Two basic types of fluorescently labeled targets, reference targets and control targets, are used to perform all of the quality control tests on catalog cDNA microarrays. An analogous combination of RNA targets and oligo targets are combined in a QC target sample used on the oligonucleotide microarrays. Reference targets specifically bind to every experimental probe printed on the cDNA microarrays, while the oligo targets specifically bind to most of the QC probes printed on the oligo QC microarray. The sample targets are labeled with both Cyanine 3 and Cyanine 5 in identical labeling reactions, combined for a self vs. self experiment, and then added to the hybridization mixture at a high concentration to yield strong fluorescent signals when bound to the features on the microarrays. Features with low signal intensity indicate problems with hybridization and are counted as defects. This test ensures that the features are present on the microarrays and capable of successful hybridization.

Spot uniformity and feature accessibility are also tested using the fluorescence data from the reference/oligo targets in the QC sample. Agilent's feature extraction software is used to analyze spot morphology of each feature on the microarray. Spots must be homogeneous, the correct size and have a round symmetrical shape (Figure 7). The software's feature non-uniformity flagger identifies spots with poor shape morphology because they might not yield accurate and reproducible data. The uniformity test is also a measure of spatial uniformity, which shows that the microarray features are accurately located on the microarray grid (Figure 8). This enables the data extraction program to easily associate each feature on the microarray with the information stored about it in the electronic layout file.

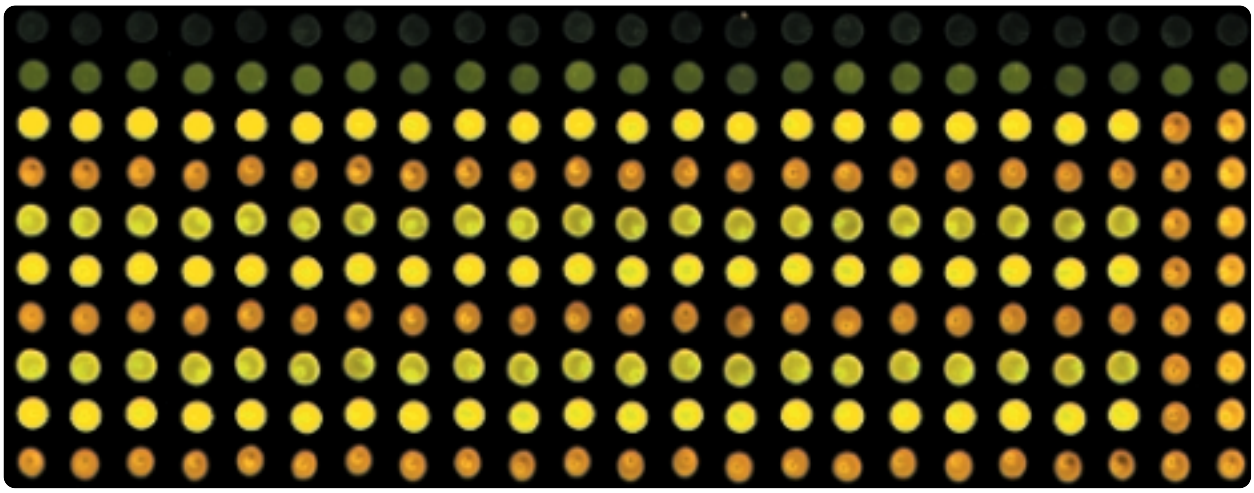


Figure 8. Features from this QC oligonucleotide microarray display a high degree of spatial uniformity and accurately fall on the microarray grid.

The precision, accuracy and sensitivity of the microarrays are measured through the use of control probes, which are fluorescence-labeled synthetic RNA probes complementary to non-mammalian control targets printed on the microarray. The molar concentration of each Cyanine 3 and Cyanine 5 control probe is known and can be spiked into the hybridization mixture at fixed ratios and concentrations in dye swap and sensitivity experiments. Unlike the reference sample probes, the control probes are not at high concentrations. This enables the sensitivity of the microarrays to be calculated using the lowest levels of detectable signal (LLD) in both the red and green channels. The average signal from the control features is calculated from the target concentration where the signal intensity is equal to the detection limit (equal to 3 times the standard deviation around background). These two measurements ensure that the microarray sensitivity is equivalent to the detection of 3 transcript copies per cell per million cells for cDNA microarrays and 1 transcript copy per cell per million cells for oligo microarrays. Even at these low levels, the signal from these features is large enough to confidently distinguish them above the background fluorescence.

Feature log ratio accuracy is determined through a comparison to the known Cyanine 3/Cyanine 5 molar ratios of the controls added into the hybridization mixture. The control features are printed multiple times on the microarrays so that mean accuracy and reproducibility can be determined for intra-microarray and inter-microarray measurements. The inter- and intra- microarray precision measurements ensure the reproducible quality of the workmanship throughout the microarray print run for every lot of microarrays printed.

Packaging and Shipping

Once the QC process is successfully completed, Agilent can guarantee with confidence that a minimum of 95% of the features printed on the microarrays are present, hybridizable, and capable of generating accurate and reproducible data. The microarrays previously set aside can now be packaged into nitrogen purged plastic boxes and vacuum packed plastic bags that will protect them from exposure to light and reactants in the air. The microarrays are accompanied with electronic layout files on a CD ROM, microarray hybridization protocols, and the "craftsmanship statement of guarantee" ensuring Agilent is confident of the quality of our work and proud to stand behind the process that went into making our microarrays.

Ordering Information

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