

Agilent Human 1 cDNA Microarray Kit Protocol

User's Guide

For use with cat. no. G4100A

Version 1.0

June 2001

Notices

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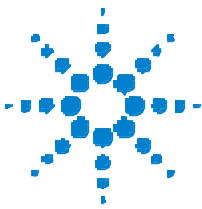
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1 Introduction

Agilent's Human 1 cDNA Microarray contains amplified clones from Incyte's sequence-verified Unigene 1 (formerly UniGEM v 2.0) and Human Drug Target (formerly the Human GEM1) DNA clone sets. These amplified cDNAs are arranged in an microarray format such that each individual feature (or spot) on the microarray corresponds to a different clone (i.e., Incyte Clone ID). Each microarray contains over 12,000 amplified cDNA's from the Incyte clone collection. Two microarrays are present on each 1"x3" glass slide. There are four microarrays in each kit.

The compact disk (CD) included in this kit, contains a design file that links a specific amplified cDNA clone with its location on the microarray. The sequence information for each clone can be obtained directly from Incyte's LifeSeq Database. Most of the DNAs on this microarray map to publicly available sequences. For these, gene name, the GenBank accession number, and the UniGene Cluster ID (if available) are also provided with the clone ID in the microarray design file on the compact disk. The clones on the microarray that do not map to any publicly available sequence are designated as Incyte EST's (Expressed Sequence Tags).

Described in this manual are procedures for use of Agilent's Human 1 cDNA Microarray Kit for the detection of genes expressed differentially between two different biological samples. This is accomplished by competitively hybridizing differentially labeled cDNA targets synthesized from two different RNA populations. Agilent recommends use of the Direct Label cDNA Synthesis Kit (cat. no. G2557A) and Perkin-Elmer/NEN cyanine 3 and cyanine 5 dCTPs (cat. no. NEL576 and NEL577, respectively - reference quotation number AG2100 for a 30% discount off of list price) for synthesis of cyanine 3- and cyanine 5-labeled cDNA targets.

Kit Contents

- 4 Human 1 cDNA microarrays (2 slides)
- cDNA Microarray Hybridization Users Guide
- CD containing feature information
- Product Data Sheet
- Microarray Slide Template
- 2x Deposition Hybridization Buffer 125 μ l

Required Equipment

- Glass cover slips, 24 x 30 mm (one/microarray-two/glass slide: Corning cat. no. 2935-243; VWR cat. no. 48396-100)
- Microarray Slide Hybridization Chamber (waterproof and compatible with coverslip hybridization)

NOTE

The design for a standard hybridization chamber can be found on the Pat Brown lab website (<http://cmgm.stanford.edu/pbrown/>). There are several commercially available hybridization chambers that work well for coverslip hybridizations. The specific design choice is the preference of the user. However, the chamber must be water-tight. We also recommend that the chamber contain a humidification reservoir to maintain chamber humidity during incubation.

- Forceps
- Micropipettors to pipette a range of 1 μ l to 1 ml volumes
- Sterile, nuclease-free 1.5 ml microcentrifuge tubes
- Sterile, nuclease-free aerosol barrier pipet tips
- Microcentrifuge
- Vortex mixer
- Ice bucket

1 Introduction

- Waterbath or heating block set to 65° C
- Waterbath or heating block set to 98° C
- Three slide staining dishes, with slide rack (Wheaton, cat. no. 900200)
- Magnetic stir plates (2)
- Magnetic stir bars (2)
- Inert dusting gas canister (recommended supplier)
- Benchtop/clinical centrifuge, with large swinging buckets that will hold slide racks vertically (for drying).
- Plastic slide holder to hold slides in centrifuge buckets during centrifuge drying

Required Reagents Not Included in Kit

- Cyanine 3- and cyanine 5- labeled cDNA (for use with Section 2.2)
- Nuclease-free water (Invitrogen, cat. no. 10977-015)
- 20x SSC (Amresco, cat. no. 0804)
- 10% SDS (Invitrogen, cat. no. 15553-027)
- Deionized water (e.g., MilliQ)
- Deposition Control Targets (Operon, cat. no. SP300)

NOTE

Prepare Deposition Control Targets by resuspending in 60 µl of DNase/RNase-free water. Store at -20° C until use.

- Human Cot-1 DNA, 1 mg/mL (Invitrogen, cat. no. 15279-011)

Safety Notes

- Observe standard laboratory safety procedures when working in the laboratory.
- The Deposition Hybridization Buffer in this kit contains lithium chloride (LiCl) and lithium lauryl sulfate (LLS).
 - LiCl is toxic and a potential teratogen. Wear gloves.
 - LLS is harmful if inhaled and is irritating to eyes, respiratory system and skin. Wear gloves, eye protection and laboratory coat.

General Procedural Notes

- To prevent contamination of reagents by ribonucleases, always wear powder-free laboratory gloves and use dedicated solutions and micro-pipettors with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- Cyanine 3 and cyanine 5 are photolabile. Minimize exposure to light.

Microarray Handling Notes and Recommendations

- There are two microarrays on each slide printed on the side opposite the barcode (i.e., the “active” side). The barcode is placed on the inactive side of the slides.
- Each microarray is printed in a 119 row by 156 column grid.

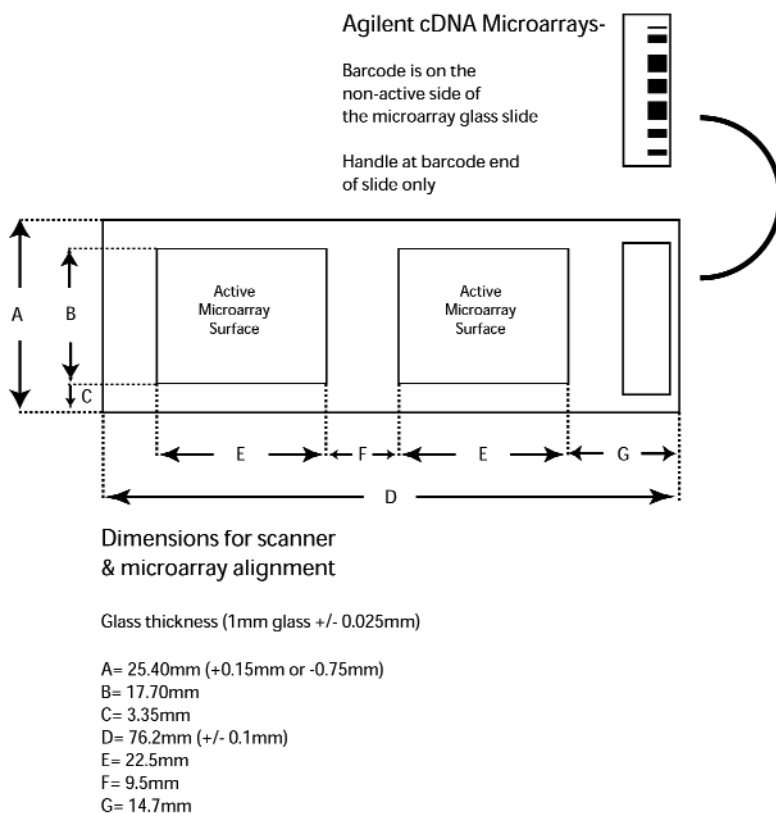
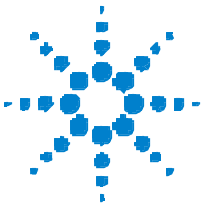


Figure 1 cDNA Microarrays

- Handle slides by their edges. Never touch the surfaces of the slides.
- Wear powder-free gloves when handling the slides
- Never let the microarrays dry out during the hybridization and washing steps



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Hybridization Procedure

RNA Preparation and Labeling

The Agilent Human 1 cDNA Microarray is compatible with the Agilent Microarray scanner designed for dual-color or competitive hybridization for monitoring gene expression. In the hybridization procedure described below, cyanine 3- and cyanine 5-labeled cDNA prepared from 20 µg total RNA are combined in a single hybridization solution.

Numerous methods and kits are available for the preparation of labeled cDNA samples for hybridization to microarrays. Most of these methods use a reverse transcriptase to convert mRNA isolated from tissues or cells of biological interest to labeled cDNA. We recommend using Agilent's Direct Labeling cDNA Synthesis Kit (cat. no G2557A), which provides the reagents and protocols to the generation of cyanine 3- and cyanine 5-labeled cDNA targets from either total or poly-A RNA. Following labeling, paired cyanine 3- and cyanine 5-labeled samples should be combined, dried, and stored at -80° C until they are needed for hybridization.

Following hybridization, scanning, and feature extraction, differences in the relative intensities of the cyanine 3 fluorescence (which fluoresces in the green region of the spectrum) and cyanine 5 fluorescence (which fluoresces red region of the spectrum) are used to measure changes in expression levels between the two samples.

Preparation of the Hybridization Solution

NOTE

To obtain an accurate assessment of differential expression, it is important to remove differences in relative intensities that may be an effect of the label (i.e., differences resulting from the two different cyanine dyes). We recommend all hybridization experiments be performed in duplicate using "dye-swapped" samples (i.e., one hybridization with sample X labeled with cyanine 3 and sample Y labeled with cyanine 5, then a second hybridization with sample X labeled with cyanine 5 and sample Y labeled with cyanine 3). Simple fluorescence intensity differences between the dyes can be normalized using most software packages. However, we have observed that the ratios for some probes on the microarray can vary depending on which samples are labeled with cyanine 3 versus cyanine 5. To correct for this dye bias, we recommend performing dye-swap hybridizations and averaging the relative expression measurements obtained for the two hybridizations.

NOTE

Human Cot-1 DNA consists of sheared DNA fragments that contain highly repetitive sequences (e.g., Alu and Kpn sequences). The addition of Cot-1 DNA to the hybridization reaction is intended to minimize signal resulting from hybridization to repetitive sequences. In hybridization experiments conducted with and without Cot-1 DNA, we have observed differences in the absolute intensities and ratios for some probes on the microarray. We have not observed similar differences with the addition of other nucleic acids, such as yeast tRNA. Thus, we recommend including Cot-1 DNA in the hybridization solution, particularly when comparing results to previously generated expression data generated using Cot-1 DNA.

- 1 Thaw Deposition Control Targeted (prepared as described in the beginning of the protocol in the Reagents Not Included in the Kit section) and Cot-1 DNA. Place on ice.
- 2 For each microarray to be hybridized, resuspend, by gentle pipetting, one tube of cyanine 3/cyanine 5-labeled cDNA from the labeling reaction in 7.5 µl of nuclease-free water. Store on ice.
- 3 To the 7.5 µl labeled cDNA add the following:
 - 2.5 µl Deposition Control Target
 - 2.5 µl Cot-1 DNA
 - 12.5 µl 2x Deposition Hybridization Buffer
- 4 Mix the hybridization mix prepared above well and incubate at 98° C for two minutes to denature the cDNA.
- 5 After incubation, centrifuge the hybridization mix for 60 seconds at room temperature, then leave the solution at room temperature until use.

Hybridization of Labeled cDNA to Slides

NOTE

It is, of course, extremely important that the solution be applied to the correct side of the slide. Carefully, place the slide on the Microarray Slide Template with the bar code down (the bar code is on the *bottom* of the slide, opposite the microarrays). In this orientation, the microarrays are now on the *top* active surface of the slide.

CAUTION

The microarray surface is very delicate. Avoid touching the microarray surface with fingers, pipet tips, etc., as these can leave marks on the surface that interfere with hybridization, detection, or feature extraction and analysis.

- 1 With a canister air duster, blow any dust or debris from the slide surfaces.

NOTE

House nitrogen may contain oil or water droplets and should not be used without a filter specifically designed to remove these contaminants.

- 2 Place each slide to be hybridized, active-side up, onto Microarray Slide Template.

NOTE

The barcode is on the side opposite the microarrays. The slide should be oriented so that the barcode is in contact with the Microarray Slide Template.

- 3 Pipet 25 μ L of Hybridization Mixture (prepared above) onto the center of each microarray, being careful to avoid introduction of air bubbles.

NOTE

It is critical to avoid touching the microarray surface with the pipet tip.

- 4 Place an air-dusted coverslip over each microarray, touching one end of the coverslip onto the glass surface and slowly lowering the other to allow the Hybridization Mixture to fill the entire surface beneath the coverslip.

NOTE

Care must be taken to avoid bubble formation. If a bubble forms in the Hybridization Mixture, it may escape on its own, or it may be possible to coax the bubble out by very carefully lifting the coverslip. However, the coverslip is extremely fragile so take extra care if this procedure is tried.

- 5 Add nuclease-free water to each humidification reservoir to minimize evaporation of the Hybridization Mixture during incubation.
- 6 Place the slide into a hybridization chamber base.
- 7 Place hybridization chamber cover on top of the base. Tightly close hybridization chamber.
- 8 Submerge in 65° C waterbath. Incubate overnight (approximately 17 hours).

NOTE

The waterbath platform must be level and the hybridization chamber must be kept level at all times after it has been closed and sealed.

Washing Slides

- 1 Prepare the following wash solutions

A Wash Solution 1: 0.5x SSC, 0.01% SDS

- i) Mix the following components in the order listed below to avoid precipitation:

Table 1 Wash Solution 1

Component	Volume (ml)
MilliQ water	974.0
10% SDS	1.0
20x SSC	25.0
Volume	1000

- ii) Pass solution through a 0.2 μ m sterile filtration unit.
- iii) Store solution at room temperature.

B Wash Solution 2: 0.06x SSC

i) Mix the following components, in the order indicated:

Table 2 Wash Solution 2

Component	Volume (ml)
MilliQ water	997
20x SSC	3.0
Volume	1000

ii) Pass solution through a 0.2 μm sterile filtration unit.

iii) Store solution at room temperature.

- 2 Prepare two staining dishes by filling with Wash Solution 1 (0.5x SSC, 0.1% SDS). In one dish, include slide rack.
- 3 Prepare a third staining dish containing Wash Solution 2 (0.06x SSC).
- 4 Remove hybridization chamber from 65° C waterbath, dry chamber, and disassemble.
- 5 Remove slide from chamber carefully using forceps while keeping the coverslip in place.

NOTE

Observe any bubbles or liquid that may have leaked onto the slide.

- 6 Remove coverslips by gently dipping the slide in Wash Solution 1 (i.e., the container that does not have a slide rack).

NOTE

It may be necessary to flutter the slide up and down gently several times to allow the coverslip to “float” off the slide. The corner of the coverslip can scratch the microarray surface while slipping off of the glass slide. Try to ensure that the coverslip slides off while parallel to the slide.

- 7 Place slide in the glass slide rack submerged in Wash Solution 1 into the second staining dish at room temperature.
- 8 Repeat steps 3-6 above for the remaining slides.

NOTE

It is recommended that you process only up to eight microarrays at one time so that the microarrays only sit in the wash solution for a few minutes before proceeding to step 9.

- 9 When all slides are submerged in Wash Solution 1, place staining dish on magnetic stirrer. Stir for five minutes with moderate stirring.
- 10 Transfer slides to a slide rack in the staining dish filled with Wash Solution 2 (0.06x SSC) at room temperature. Stir for two minutes with moderate agitation.
- 11 Carry the dish with the microarray slides to the centrifuge to be used in the next step.
- 12 Quickly transfer slides from Wash Solution 2 into plastic racks for drying by centrifugation. Place slides in swinging buckets in benchtop (clinical) centrifuge. Centrifuge for two minutes at 400 g at room temperature to dry the slides.

NOTE

This step must be performed as quickly as possible following the wash step; if the slides are allowed to air dry prior to centrifuging, a permanent residue will form on parts of the slides, making them unreadable. (The residue typically shows up as bright green areas on the scanned images.) It is therefore important to have the centrifuge racks and buckets set up in advance so that transfer from wash solution into the centrifuge occurs as quickly as possible.

- 13 Scan the slides and store them in a polypropylene slide box in the dark to prevent irreversible photobleaching of the cyanine dyes. We recommend storage in a vacuum dessicator or use of a N₂ purge box.

Generating Images of the Hybridized Microarrays

There are several commercially available scanners available on the market. To detect the hybridized cyanine 3- and cyanine 5-labeled cDNA, use a slide scanner capable of exciting and detecting the fluorescence from the cyanine 3 and cyanine 5 fluorescent molecules (532 and 633 nm laser lines). We recommend using Agilent's dual-laser Microarray Scanner (G2565AA) which has dynamic autofocusing for high resolution feature scanning and for high-throughput analysis of multiple slides. To use non-Agilent scanners, refer to the manufacturer's instructions.

The diagram in the "Microarray Handling Notes and Recommendations" section provides information concerning the positions of the microarrays on Agilent slides which may be useful when using non-Agilent scanners:

In the scanned image, repeated sets of control probes should be visible in the first and last row on each corner of the microarray.

The information for each probe on the microarray is contained in the files included on the compact disk. There are .xml files designed for use with Agilent's Feature Extraction Software (provided with the Agilent Microarray Scanner), and tab-delimited text files (.txt files) for use with other software packages. The "design" files contain the information about each probe arrayed and the "defect" files list the probes that did not print properly on this microarray batch. For detailed information describing each file, use of the files with Agilent's software, and a description of the structure of each file for use with non-Agilent software, refer to the "README.txt." file on the disk.

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