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Comparative Genomic Hybridization: DNA labeling, hybridization and detection

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Abstract

Array-CGH involves the comparison of a test to a reference genome using a microarray composed of target sequences with known chromosomal coordinates. The test and reference DNA samples are used as templates to generate two probe DNAs labeled with distinct fluorescent dyes. The two probe DNAs are co-hybridized on a microarray in the presence of Cot-1 DNA to suppress unspecific hybridization of repeat sequences. After slide washes and drying, microarray images are acquired on a laser scanner and fluorescent intensities from every target sequence spot on the array are extracted using dedicated computer programs. Intensity ratios are calculated and normalized to enable data interpretation. Although the protocols explained in this chapter correspond primarily to the use of large-insert clone microarrays in either manual or automated fashion, necessary adaptations for hybridization on microarrays composed of shorter target DNA sequences are also briefly described.

Keywords

probe labeling; random priming; hybridization; detection; Comparative Genomic Hybridization; array-CGH

1. Introduction

Array-CGH was developed in the late nineties (1,2) to detect DNA copy number changes at high resolution along the genome or locus of interest (*see Chapter 3 for a general introduction on the method*). This chapter details the methods employed to label, hybridize and detect genomic DNA probes for array-CGH.

As a first step, two genomic DNA samples - one test and one reference - are labeled separately using two different fluorescent dyes: generally Cyanine 3 (Cy3; excitation/emission wavelength maxima: 550/568nm) and Cyanine 5 (Cy5; 650/668nm). The probe DNA is commonly generated by enzymatic incorporation of Cy3 or Cy5 labeled nucleotides (usually dCTP or dUTP). Several strategies are possible for genomic DNA labeling, such as nick translation (1,3), direct labeling PCR (4) or random priming (5). Nick translation requires relatively high quantities of input DNA (usually 2µg), which can be problematic when working with small and/or precious DNA samples. PCR labeling overcomes this problem, as DNA is amplified and labeled in the same reaction. However, the exponential amplification of genomic DNA can bias the representation of the target by the amplified probe, thus leading to artifactual ratio variations after hybridization (4). Random primed labeling represents a good compromise between the two first techniques: (1) it requires moderate quantities of input DNA (usually 50 to 500 ng); (2) the representation of target sequences is not biased in the resulting probe, as the reaction consists of a moderate linear amplification of the template DNA by the Klenow fragment, using a mix of random

hexanucleotides as primers. The probe DNA can also be labeled by chemical techniques such as cis-platinum labeling (6), which is based on the capability of monoreactive cisplatin derivatives to react at the N7 position of guanine moieties in DNA (7). However, cis-platinum labeling requires more than one microgram of template DNA as the chemical reaction does not create new DNA molecules.

After DNA labeling, both probes are co-precipitated and dissolved in hybridization buffer. The composition of this buffer, combined with the temperature of the hybridization, is critical to enable efficient and specific hybridization of the probes to the target DNA printed on the microarray. The hybridization buffer used in these protocols contains 50% of formamide, 0.1% of Tween20 and 5 to 10% of dextran sulfate. Formamide acts as a denaturing agent, which increases the hybridization stringency and prevents unspecific hybridization at lower temperatures such as 37°C. Tween20 is a nonionic detergent which minimizes non-specific fluorescence background on the surface of the slide. Dextran sulfate is a neutral component which consists in polymers of anhydroglucose in aqueous solutions: in homogeneous solution, it excludes DNA from the volume occupied by the polymer. In consequence, DNA concentration is “artificially” increased, which improves hybridization kinetics (8).

Hybridization to the array is performed at 37°C in the presence of Cot-1 DNA. Cot-1 DNA is the fraction of genomic DNA consisting largely of highly repetitive sequences. It is obtained from total genomic DNA by selecting for the most rapidly re-associating DNA fragments after denaturing. A large excess of Cot-1 DNA suppresses the hybridization of high-copy repeat sequences that are present in labeled DNA probes and thus prevents their hybridization to the corresponding repeat sequences that are also present in the target DNA (9,10).

After hybridization, slides are washed several times in PBS/0.05% Tween20 to remove excess hybridization buffer and reduce nonspecific background signal at the surface of the slide. The most critical wash is performed at 42°C in 50% formamide/2x SSC (or at 54°C in 0.1x SSC in the automated protocol); this stringent wash is essential for the selective and efficient elimination of probe fragments that are not hybridized specifically to the target arrayed DNA.

After washes and slide drying, microarray images are acquired for data analysis. Array-CGH was originally developed from CGH on chromosomes, a method in molecular cytogenetics using fluorescence microscopy (11,12). In consequence, the first acquisition systems used a CCD camera coupled to 0.5x or 1x magnification optical system (1) or a confocal laser scanning microscope (2). Today, with the huge expansion of microarray technologies, many scanners specific for DNA microarrays have become available that enable quick and easy image acquisition. The resolution of these scanners is typically 5 to 10 microns per pixel. Images are usually saved under Tagged Image File Format (TIFF), which is compatible with many distributed image analysis programs.

The methods below describe in detail how to label probes by random priming and how to hybridize and detect them on CGH microarrays (in particular on large-insert clone microarrays constructed using methods in **Chapter 16**) by either manual or automated procedures. Array-CGH profiles can then be produced from array images and interpreted using strategies described in **Chapter 3**.

2. Materials

2.1. Probe preparation

1. BioPrime Labeling Kit (Invitrogen): contains 2.5x random primers solution, water, Klenow fragment and stop buffer.
2. dNTP mix (10x): 1mM dCTP, 2mM dATP, 2mM dGTP and 2mM dTTP in TE buffer.
3. 1mM Cy3-dCTP (NEN Life Science).
4. 1mM Cy5-dCTP (NEN Life Science).
5. Microcon YM30 columns (Millipore/Amicon).

2.2. Hybridization and detection, manual protocol

1. Hybridization buffer M: 50% formamide (deionized, available from Sigma), 10% dextran sulfate, 0.1% Tween 20, 10mM Tris-HCl, 2x SSC, pH 7.4. Store at -20°C .
2. 3M Sodium acetate, pH 5.2.
3. Human Cot-1 DNA (Invitrogen).
4. Formamide.
5. 2x SSC, pH 7.4.
6. PBS / Tween 20 0.05%, pH 7.4.
7. 0.1x SSC, pH 7.4.
8. Cover slip.
9. Hybridization chamber.

2.3. Hybridization and detection, automated protocol

1. Hs.400PRO/4800PRO automated slide processing station with 51x20mm hybridization chambers (Tecan, Inc).
2. Cysteamine (available from Sigma, see Note 1).
3. Hybridization buffer A: 50% formamide (deionized), 7.5% dextran sulfate, 0.1% Tween 20, 10mM Tris-HCl, 2x SSC, pH 7.4. Store at -20°C .
4. 3M Sodium acetate, pH 5.2.
5. Human Cot-1 DNA (Invitrogen).
6. PBS / 0.05% Tween 20, pH=7.4.
7. 0.1x SSC, pH=7.4.

¹Cy3 and Cy5 are the most commonly used fluorochromes for DNA labeling. However, both molecules are very sensitive to environmental conditions such as light, high ozone and humidity levels (13). To avoid any problem of dye degradation or fading, all experiments from DNA labeling to slide scanning should be performed in a laboratory with controlled temperature (20-25°C), relative humidity (25-35%) and ozone level (<0.02ppm). Alternatively, to limit premature degradation of Cy3 and Cy5, an anti-oxidant, such as cysteamine, can be added to hybridization buffers A and M (cysteamine 10mM) as well as PBS / 0.01% Tween 20 (cysteamine 2mM).

3. Methods

Quantities and volumes described here correspond to the use of 2×3cm microarrays or Tecan Hs. PRO 51×20mm hybridization chambers: they should be rescaled when using arrays or chambers with different dimensions. The protocols were developed for the use of large-insert clone microarrays, constructed following the protocols described in **Chapter 16**. However, this protocol has been applied successfully to microarrays composed of smaller target DNA sequences, such as small-insert clones (size ranging from 1 to 4kb) or PCR products (150bp to 1kb), by following the automated procedure with slight modifications as described in the footnote of Table 1.

3.1. Probe preparation

1. Prepare two separate 1.5ml tubes for the test and the reference DNA samples. In each tube, add 150ng of test or reference genomic DNA, 60μl of 2.5x random primers solution and water to a final volume of 130.5μl (*see Note 2*).
2. Denature the samples for 10min at 100°C, and immediately cool on ice. Then add into each tube, still on ice: 15μl of 10x dNTP mix, 1.5μl of Cy3 or Cy5 labeled dCTP and 3μl of Klenow fragment (*see Note 1*).
3. Incubate the reactions at 37°C overnight.
4. Stop the reactions by adding 15μl of stop buffer.
5. The next step is the removal of unincorporated labeled nucleotides using Microcon YM30 columns. Put the columns onto the tubes provided by the suppliers. Then add the entire labeled test and reference samples to 140μl of water on top of the columns (one column per sample).
6. Centrifuge for 5 min at 12,000g. Discard the flow-through, put the columns (which should contain the labeled probes) back onto the tubes and add 300μl of water to the columns.
7. Spin again for 5 min at 12,000g. Discard flow-through, add 50μl of HPLC water to the columns and place filter upside-down onto a fresh tube.
8. Centrifuge for 2 min at 2,000g. The solution collected at this step should be colored in blue for Cy5 labeling, in pink for Cy3 labeling (*see Note 3*).

3.2. Hybridization and detection, manual protocol

1. Add the following solutions into a 1.5ml tube: Cy3 and Cy5 labeled and cleaned test and reference DNA samples (the volume should be ~100μl per sample); 135μl of human Cot1 DNA (1μg/μl); 35μl of Sodium acetate 3M; 1ml of ethanol (*see Note 4*).
2. Mix and precipitate at –20°C overnight or at –70°C for 30 min. Centrifuge precipitated DNA for 30 min at 4°C, at maximum speed (~16,000g).

²The quality and purity of DNA samples used as templates for DNA labeling should be carefully monitored. Genomic DNA should show no degradation (by electrophoresis on agarose gel; *see Fig. 1A*) and no protein contamination (on spectrophotometer, 280/260 ratio should be greater than 1.8; *see Fig. 1B, 1C*). The use of DNA samples which do not fulfill these quality criteria may result in failure of the labeling reaction or low quality of the array-CGH results (i.e. higher technical variability of the array-CGH profile impairing the detection of copy number changes).

³DNA probe quantity and quality should be controlled after removal of unincorporated labeled nucleotides. First, 3μl of each sample collected in step 8 should be run on a 2.5% agarose gel to check for the presence of a DNA smear with most fragments below 500bp (*Fig. 1A*). In addition, probe DNA concentration as well as Cy3 or Cy5 incorporation can be measured by using only 1μl of each collected sample using a NanoDrop Spectrophotometer (*Fig. 1B, 1C*).

3. Wash pellets in 1ml ethanol 80% and centrifuge again for 5 min at maximum speed. Remove supernatant and dry the pellets. Re-dissolve the pellets in 35µl of hybridization buffer M.
4. Denature the labeled probes for 10 min at 70°C then incubate for 60 min at 37°C.
5. Apply 30µl of the hybridization solution onto the array and cover with a 2.4×3.6cm cover slip. Place the slide into a hybridization chamber previously humidified with 2x SSC / 20% formamide. Seal the chamber and incubate at 37°C for 24-48h.
6. Remove the cover slip by placing the slide into a tall glass trough containing PBS / 0.05% Tween 20 to wash off the hybridization solution
7. Transfer the slide into a new glass trough and wash in PBS / 0.05% Tween 20 for 10 min at room temperature under quick agitation.
8. Place the slide into a pre-heated 50% formamide/2x SSC solution and incubate for 30 min at 42°C under slow agitation.
9. Transfer the slide into fresh PBS/0.05% Tween 20 and wash again for 10 min at room temperature under quick agitation.
10. Dry the slide by centrifugation at 150g for 1 min, then store it in a light-proof box and scan as soon as possible (see Note 6).

3.3. Hybridization and detection, automated protocol

The manual protocol described in **paragraph 2.2** enables the processing of up to 8 slides per person per day. For high-throughput array-CGH analysis, we have adapted the protocol for the use of automated hybridization stations (Hs400PRO/Hs4800PRO; Tecan, Inc). The Hs4800PRO station enables one person to process 12 slides per unit per day, and can be configured with up to 4 independent 12-position units.

There are two main changes in the automated procedure: (1) The hybridization buffer A contains only 7.5% of dextran sulfate: this reduces the viscosity of the solution and makes it compatible with injection and mixing in the hybridization chambers; (2) the second wash - with 50% formamide/2x SSC at 42°C for 30 minutes - has been replaced by washes with 0.1x SSC at 54°C, to preserve the components of the hybridization station. The Hs. PRO station should be programmed for array-CGH as described in Table 1, before starting Step 4 of the protocol below (see Note 5).

⁴One critical factor for the success of array-CGH is the efficient suppression of repeated sequences by the Cot-1 DNA during hybridization (see **Introduction**). We have noticed that commercially available Cot-1 DNA tends to show batch to batch variations in terms of suppression efficiency. Suppressive hybridization with high quality Cot-1 DNA results in log2ratio values close to 0.6 for single copy gains and -1 for single copy losses. The use of lower quality Cot-1 DNA may result in incomplete repeat suppression, compressed abnormal ratios and increased background ratio variability.

⁶Array images are generally acquired using a commercial DNA microarray scanner. We routinely use the scanner from Agilent technologies: this fully-automated system (with a 48-slide loading carousel) uses dynamic auto-focus to keep features in focus while scanning. Scanners can also be purchased from companies such as Perkin Elmer, Tecan or Axon Instruments. There are several software solutions available for image quantification (see *example of microarray image* in Fig.2A). Although spot intensity extraction programs are usually supplied with commercially available scanners, they can be purchased separately. The usual commercial programs include GenePix (Axon Instruments), ScanArray (Perkin Elmer), Agilent Feature extraction software (Agilent Technologies) and Bluefuse for microarrays (BlueGnome Ltd). Other programs are freely available, such as TIGR Spotfinder (14) (<http://www.tm4.org/spotfinder.html>) or UCSF Spot (15) (<http://www.jainlab.org/downloads.html>). For further data analysis (described in **Chapter 3**), the ratio values between the fluorescent intensities in both channels on every spot on the array are calculated (usually after the subtraction of local background fluorescence). Intensity ratios are then normalized, for example by dividing each individual ratio by the median ratio value of all clones. The normalized ratio for each clone is then plotted against its position along the genome (see *example of normalized array-CGH profile* in Fig. 2B).

⁵The hybridization stations Hs.400PRO/4800PRO replace two other stations, which are still available: Hs400 and Hs4800. These two non-PRO stations can also be used for array-CGH. In this case, as the mixing system is different in the two non-PRO stations, the hybridization buffer A should contain only 5% of dextran sulfate instead of 7.5% in order to reduce the viscosity of the solution.

1. Add the following solutions into a 2 ml tube (named Tube A): Cy3 and Cy5 labeled and cleaned test and reference DNA samples (the volume should be ~100µl per sample), 135µl of human Cot1 DNA (1µg/µl), and 35µl of Sodium acetate 3M and 1ml of ethanol.
2. Into another 2ml tube (named Tube B), add 100µl of Herring Sperm DNA (10µg/µl), 10µl of Sodium acetate 3M and 300µl of ethanol.
3. Mix both tubes and precipitate DNA at -70°C for 30min or at -20°C overnight.
4. Centrifuge for 30 min at 4°C, at maximum speed (~16,000g).
5. Remove supernatants and wash pellets in 1 ml ethanol 80%. Centrifuge again for 5 min at 16,000g. Remove supernatants, dry pellets and re-dissolve them in 120µl of hybridization buffer A.
6. Denature DNA in Tubes A and B for 10 min at 70°C.
7. Keep Tube A at 37°C. Start the array-CGH program on the Tecan station and inject 100µl of the hybridization solution from Tube B into the corresponding slide position following the instructions displayed on the machine.
8. After ~ 45 min, inject 100µl of the hybridization solution from Tube A into the corresponding slide position following the instructions displayed on the machine.
9. When the array-CGH program is completed, place the slide in a light-proof box and scan as soon as possible (see Note 6).

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Figure 1. Quality control of genomic probes after random primed labeling

(A) Pattern of DNA samples on a 2.5% agarose gel, before and after labeling by random primed labeling. The test and Reference DNA (Positions 1-2) samples show no sign of degradation before random priming. After the labeling reaction, each probe labeled with Cy3 (3-4) or Cy5 (5-6) consists of a DNA smear with most fragments below 500bp. MW: molecular weight marker. (B and C) Measurement of DNA concentration and dye incorporation on a NanoDrop spectrophotometer. All samples give a 260/280 ratio above 1.8, indicating that there is no residual protein contamination before and after labeling and cleanup. The graph (B) displays the 1mm absorbance of each sample according to the light wavelength in nm. Test and reference genomic DNA show one single pick at 260nm, while each probe labeled with Cy3 or Cy5 shows a second pick with maximum absorbance at 550nm and 650nm, respectively. The table (C) reports Cy3 or Cy5 incorporation rates for each labeled probe. PSA is the probe specific activity: $PSA = (\text{pmol of dye per ul}) / (\text{ug of DNA per uL})$. It reports the dye incorporation rate and should be greater than 25pmol/ μg of DNA with a DNA yield (which is the quantity of synthesized probe) greater than 8 μg .

Figure 2. Example of microarray image and genome profile after array-CGH

(A) Merged images displaying signal intensities after comparative genomic hybridization of one test female DNA (labeled with Cy3, in green) versus one reference male DNA (labeled with Cy5, in red) on a microarray covering the entire human genome with ~30,000 large-insert clones (16). The microarray (left panel) is divided in 8×4 blocks, each of them being composed of 31×31 spots (right panel). As both DNA samples are from healthy anonymous individuals, most spots (clones) show no copy number changes and appear in yellow (green = red = 2 DNA copies). However, on each block (right panel), a few clones appear in green or in red: most of them are clones mapped on chromosome X (green = 2, red = 1) or on chromosome Y (green = 0, red = 1). (B) Whole genome array-CGH profile derived from the images merged in (A). For each spot, the log₂ratio value is calculated, normalized by the median of all log₂ratio values and plotted against the genome position of the corresponding clone. The profile shows log₂ratio values that are close to zero on autosomes, increased (up to +1) on chromosome X and decreased (down to less than -3) on chromosome Y. Some local log₂ratio variations indicate the presence of small copy number changes between the 2 individuals (examples indicated by red arrows).

Table 1
Array-CGH program for Hs. PRO stations (Tecan, Inc)

PROTOCOL			
<i>Step</i>	<i>Type</i>	<i>Temp.</i>	<i>Options</i>
1	Wash	37°C	First: Yes, Channel: 1, Runs: 1, Wash time: 0:00:30, Soak time: 0:00:00
2	Injection	37°C	Agitation after Injection: Yes
3	Hybridization	37°C	Agitation Frequency: High, High Viscosity Mode: Yes, Time: 0:45:00
4	Injection	37°C	Agitation after Injection: Yes
5	Hybridization	37°C	Agitation Frequency: High, High Viscosity Mode: Yes, Time: 21:00:00 *
6	Wash	37°C	First: No, Channel: 1, Runs: 14, Wash time: 0:00:30, Soak time: 0:00:30
7	Wash	54°C *	First: No, Channel: 2, Runs: 5, Wash time: 0:01:00, Soak time: 0:02:00
8	Wash	25°C	First: No, Channel: 1, Runs: 7, Wash time: 0:00:30, Soak time: 0:00:30
9	Wash	25°C	First: No, Channel: 3, Runs: 1, Wash time: 0:00:30, Soak time: 0:00:00
10	Slide Drying	25°C	Time: 0:02:30, Final Manifold Cleaning: Yes, Channel: 5

LIQUID USAGE	
<i>Liquid Channel</i>	<i>Wash solution</i>
1	PBS / Tween 20, 0.05%
2	0.1x SSC
3	water

* For the use of microarrays composed of short target DNA sequences: (1) the time of hybridization should be extended to 48-72 hours; (2) the temperature for washes in step 8 should be 52°C instead of 54°C.