Video Article

Array Comparative Genomic Hybridization (Array CGH) for Detection of Genomic Copy Number Variants

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Abstract

Array CGH for the detection of genomic copy number variants has replaced G-banded karyotype analysis. This paper describes the technology and its application in a clinical diagnostic service laboratory. DNA extracted from a patient's sample (blood, saliva or other tissue types) is labeled with a fluorochrome (either cyanine 5 or cyanine 3). A reference DNA sample is labeled with the opposite fluorochrome. There follows a cleanup step to remove unincorporated nucleotides before the labeled DNAs are mixed and resuspended in a hybridization buffer and applied to an array comprising ~60,000 oligonucleotide probes from loci across the genome, with high probe density in clinically important areas. Following hybridization, the arrays are washed, then scanned and the resulting images are analyzed to measure the red and green fluorescence for each probe. Software is used to assess the quality of each probe measurement, calculate the ratio of red to green fluorescence and detect potential copy number variants.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51718/>

Introduction

It has been known for many years that deletions or extra copies of chromosomal segments cause intellectual disability, dysmorphism and congenital malformations, and in some cases cause genetic syndromes¹. However, the only technology available until the mid-2000s for the genome-wide detection of these changes was G-banded chromosome analysis, which has a resolution of around 5-10Mb, depending on the region and nature of the imbalance, and which cannot detect abnormal chromosomes where material has been replaced by a region from a different chromosome with the same banding pattern. Ancillary cytogenetic techniques such as fluorescence *in situ* hybridization and multiplex ligation-specific probe amplification have been available for the interrogation of specific loci, in cases of suspected specific syndromic imbalance, but it was not until the introduction of array comparative genomic hybridization (array CGH) into routine clinical diagnostic service²⁻⁵ that genome-wide detection of copy number variants (CNVs) became possible at a greatly increased resolution (typically around 120kb). Clinical service work alongside research studies have shown that CNVs for some regions are widespread in the normal population⁶⁻⁷, whilst other CNVs, previously undetectable, are associated with neurodisabilities such as autism and epilepsy⁸⁻¹¹.

The protocol described in this paper is used in our UK National Health Service (NHS) clinical diagnostic laboratory; we use novel hybridization strategies, batch testing and robotics to minimize cost in this state-funded service.

Prior to the protocol detailed below, high quality DNA should be extracted from the appropriate starting material, commonly blood, cultured cells or tissue samples. Spectrophotometry can be used to measure concentration (should be >50 ng/ul) and check 260:280 absorbance ratios (should be 1.8-2.0). Gel electrophoresis can be used to check that the DNA is of high molecular weight without significant degradation.

This protocol is designed for higher throughput laboratories that are labeling 96 samples per run using automated liquid handling robotics. However, it may be adapted for lower throughput labs without automation.

Protocol

1. Labelling Reaction

1. Prior to use, pre-aliquot cyanine 3 and 5-labeled dUTPs, unlabeled nucleotides and random primers at the manufacturer-recommended concentrations into 96-well plates and store at -20 °C ready for use. For the purposes of this protocol, aliquot 10.5 µl of the appropriate labeled dUTP and 10.5 µl unlabeled nucleotides and random primers to each well.
2. Thaw the ready-to-use 96-well plate of nucleotides and primers at 4 °C for about 1 hr, protected from light.

3. Once thawed, equilibrate this plate at RT for at least 30 min, protected from light.
4. Equilibrate DNA samples at 60 °C for at least 15 min.
5. Using a liquid handling robot, dispense nuclease-free water to each well of a 96-well plate.
NOTE: The volume of water will depend on the concentration of each input DNA sample and should be sufficient to result in a final concentration of 50 ng/μl.
6. Using a liquid handling robot, pipette 1 μg of DNA into a well of the 96-well plate (see 1.5) to bring the total volume to 20 μl.
NOTE: Smaller quantities of DNA may be used although the quality of resulting data may be compromised (for precious samples, input DNA quantities down to 400 ng can be used).
7. Using a liquid handling robot, transfer 20 μl of the equilibrated nucleotides and primers into each well of the plate of diluted DNA.
8. Seal the 96-well plate with strip caps (a tight seal is crucial).
9. Denature the DNA at 99 °C for 10 min in a PCR machine with a heated lid and then snap cool on ice for 5 min to anneal primers.
10. Using a liquid handling robot, add 10 μl of Klenow Exo DNA polymerase enzyme to each DNAs and pipette mix thoroughly.
NOTE: The total volume should be 50 μl.
11. Seal the 96-well plate and incubate at 37 °C for 16 hr.
NOTE: A shorter incubation of 4 hr is sufficient however an O/N incubation can be more convenient.
12. Using a liquid handling robot, add 5 μl of stop buffer to each well.
NOTE: A summary of the reagents used for the labeling reaction is shown in **Table 1**.

2. Removal of Unincorporated Nucleotides

1. Remove unincorporated nucleotides using silica-membrane based spin columns and a dedicated spin column processing robot (the spin columns may also be processed manually). Follow the manufacturer's instructions.
 1. Transfer the contents of each well to a 2 ml tube; pre-label these with well ID's.
NOTE: This step can be performed manually or preferably using a liquid handling robot.
 2. If using a spin column processing robot, then load the robot with 2 ml tubes, DNA purification spin columns and associated buffers.
 3. Add 250 μl high salt, DNA binding buffer (buffer PB) to the labeling reaction to bind the labeled DNA to the silica membrane.
 4. Remove impurities by washing the membrane twice with 500 μl wash buffer (buffer PE).
 5. Add 15 μl low salt elution buffer (buffer EB) to the membrane to recover the purified, labeled DNA in a volume of ~12 μl.

3. Combine Hyb Pairs

1. Combine the appropriate pairs of cyanine 3 and 5-labeled DNA, COT-1 DNA, a manufacturer-supplied blocking mix and a manufacturer-supplied hybridization buffer using a liquid handling robot.
NOTE: These hyb pairs can be a sample and a reference, or sample vs sample if shared CNVs are not an issue (e.g., we routinely pair phenotype-mismatched samples, such as a renal dysplasia patient vs a craniosynostosis patient, as they are very unlikely to carry the same clinically significant CNV). If using a reference DNA, a commercial DNA supply is recommended, and it should be processed alongside the sample.
 1. Using a liquid handling robot, add COT-1 DNA (3333 ng/μl), the manufacturer-supplied blocking mix and hybridization buffer to each well of a 96-well plate so that each well contains 1.1 μl COT-1 DNA, 4.95 μl blocking mix and 24.75 μl hybridization buffer.
 2. Add 9.35 μl of the appropriate cyanine 3-labeled DNA to each well. Pre-wet each tip to enhance pipetting accuracy.
 3. Add 9.35 μl of the appropriate cyanine 5-labeled DNA to each well. Pre-wet each tip to enhance pipetting accuracy.
 4. Seal the 96-well plate and vortex for 1 min. Spin the 96-well plate to collect contents at the bottom of each well.

4. Hybridization

1. Preheat a hybridization oven to 65°C and prewarm one backing slide and one hybridization chamber for each array slide.
 1. Denature the labeled DNA in a pre-hybridization incubation before being applying to the arrays. Perform hybridization in a rotating oven for 24 hr.
 2. Incubate the 96-well plate at 70 °C for 30 min and then leave at 37 °C for at least 5 min.
 3. Working on a heated platform at 42 °C, load each backing slide into a hybridization chamber prior to use. Ensure that the transparent part of the gasket slide aligns with the 'windowed' part of the hybridization chamber.
 4. Pipet 42 μl of the hybridization mix prepared previously (see section 3) onto the appropriate position on the array backing slide. Pre-wet each tip to enhance pipetting accuracy. Pipet slowly onto the center of each position, making sure the liquid does not touch the rubber ring boundary.
 5. Once all positions on the backing slide are filled, carefully lower the array slide on it and assemble the hybridization chamber. Make sure that the side of the array with writing on it is facing the backing slide. Be sure to tighten the hybridization chamber screw fully.
 6. Inspect the assembled hybridization chamber. Ensure that there is no leakage of hybridization mix outside the rubber ring boundaries and each air bubble is ~4 mm in height when the hybridization chamber is rested on its end vertically. Rotate the hybridization chamber and check that there are no air bubbles that are stuck in position and not moving. If there are any of these, give the chamber a sharp tap on the bench. Check that all air bubbles are moving.
 7. Place the hybridization chamber into the rotating oven at 65 °C for 24 hr. Make sure the oven rotor is balanced; use other empty chambers if necessary.

5. Washing and Scanning Slides

1. Wash arrays to remove excess labeled DNA and then scan to measure fluorescence of each probe.
 1. Take hybridization chambers out of oven and disassemble. The array and gasket slides will be stuck together; submerge these in wash buffer 1 and use a pair of plastic, flat edged forceps to pry them apart.
 2. Discard the gasket slide and place the array slide into a rack submerged in buffer 1.
 3. Wash the array slide in ~700 ml of fresh wash buffer 1 for 5 min, stirring vigorously (use a flea and a magnetic stirrer).
 4. Transfer the array slide to ~700 ml wash buffer 2 and wash for 90 sec, stirring vigorously. Gently lift the array slides out of wash buffer 2, they should emerge dry.
 5. Load the array slide into a slide holder of the scanner, using a slide protector. Load the slide into the array scanner and scan following the array manufacturer's instructions.

Representative Results

Each probe on a hybridized array is visualized as a mixture of red and green fluorochromes (see **Figure 1**). The ratio of red to green fluorescent signal for each probe is quantified by the scanner and the associated software plots these as log₂ ratios according to their genomic position, and identifies regions falling outside preset boundaries. The resulting array traces allow interpretation of regions identified as genomically unbalanced. For instance, the trace from a child with Williams syndrome, a recurring microdeletion syndrome mediated by low copy repeats in the proximal region of chromosome 7, is illustrated in **Figure 2**. This imbalance was identified by the software and indicated by a red line.

Probe fluorescent log ratios should cluster closely around 0, as shown in **Figure 2**, indicating a green/red ratio of 1:1 for normal regions of the genome. Scattered array traces may result in inaccurate calling of abnormal regions, or failure to identify genomic imbalance (see **Figure 3**). Such scatter may be caused by a number of factors, including poor DNA quality, or the presence of raised levels of ozone in the atmosphere. Monitoring of ozone levels is recommended, and where elevated ozone levels are problematic, dedicated ozone exclusion cabinets are available; use of these cabinets generally results in markedly improved array quality.

Figure 1. Image of an array following hybridization. The white box shows a magnified area, where red probes and green probes can be visualized (indicating imbalance for the regions represented by these probes), against a background of yellow probes (indicating balanced genomic regions).

Figure 2. (A) Example trace for chromosome 7 in a sample with a 1.7Mb deletion, shown by an average ratio of -0.8 for 43 consecutive probes. The deleted region in this case is associated with Williams-Beuren syndrome, consistent with the referral indication of dysmorphic features, a heart defect and intellectual disability. (B) The deleted region above displayed in the UCSC genome browser, showing the genes within the deleted region. [Please click here to view a larger version of this figure.](#)

Figure 3. Example of a scattered array trace for chromosome 7 in a sample with poor hybridization. [Please click here to view a larger version of this figure.](#)

Reagent	Volume (μl)
Primers & reaction buffer	20
DNA (1μg) + water	20
Klenow Exonuclease DNA polymerase	10

Table 1. Summary of the reagents used for the labeling reaction.

Discussion

Array CGH will not detect balanced rearrangements or ploidy abnormalities such as triploidy. Furthermore, low level mosaic imbalances may not be detected. However, array CGH has a higher resolution for CNV detection than G-banded chromosome analysis which it has replaced in many cytogenetics laboratories. It is therefore the current gold standard for genome-wide CNV detection. It may be replaced by next-generation sequencing technologies in the future but currently, their cost and the technical complexities associated with using short reads for CNV detection mean that this is not yet a good fit for clinical use.

The protocol described here is in routine use in our clinical service laboratory. Two runs of 96 samples each are processed each week using a liquid handling robot and a dedicated silica membrane spin column processing robot. Automation is highly recommended to improve consistency and maintain quality. DNA extracted from blood, saliva, prenatal samples (e.g., chorionic villi or amniotic fluid) or tissue samples can be, and routinely is, used with this protocol.

Ozone is known to degrade cyanine dyes and therefore ozone monitoring and protection is recommended. Seasonal variation in ozone levels is also common. Our laboratory monitors and records ozone levels continuously. A wall-mounted ozone removal unit is used to reduce ozone levels and particularly sensitive parts of the protocol (i.e., washing and scanning arrays) are performed in ozone-free hoods. If possible, ozone levels are kept below 5 ppb.

Most reagents are bought as kits from manufacturers to effectively outsource quality control; this has proved to be an effective strategy. However, this does not mean that quality control is not required. Indeed, quality metrics are carefully monitored for any anomalies: derivative standard deviation of log2 ratios (DLRS) should be below 0.2, cyanine 3 and 5-signal intensities should be greater than 500 and cyanine 3 and 5-signal

to noise ratios should be >30. These metrics are generated as part of the scanning protocol by the scanning software and are recorded for long term monitoring.

Disclosures

The authors have nothing to disclose.

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