

Comparative Genomic Hybridization Using DNA From Laser Capture Microdissected Tissue

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Summary

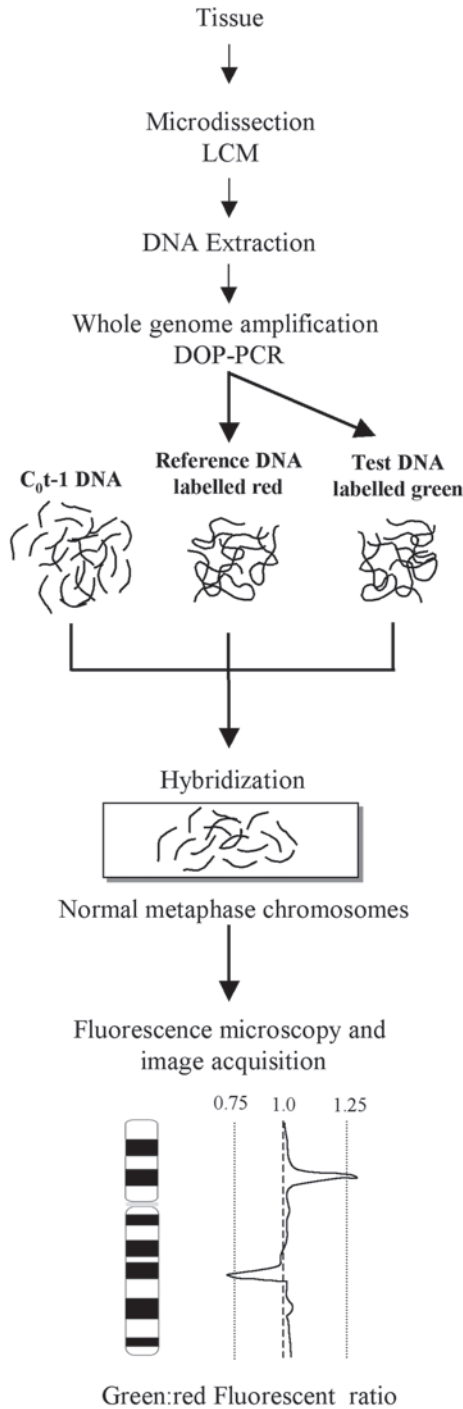
Comparative genomic hybridization (CGH) is a powerful screening technique that can identify regions of gain and loss within the whole genome in a single experiment. The combination of laser capture microdissection, whole-genome amplification, and CGH permits genomic screening with high specificity and sensitivity. This complement of techniques has enabled analysis of focal regions and subpopulations of cells within a tissue, which has previously been difficult, providing insight into disease progression and heterogeneity. This chapter outlines the techniques involved in producing labeled probes from DNA extracted from laser capture microdissected material and the methods for hybridization of these probes to metaphase chromosomes. This protocol can also be applied to the preparation of probes for CGH arrays.

Key Words: Laser capture microdissection (LCM); comparative genomic hybridization (CGH); degenerate oligonucleotide primed PCR (DOP-PCR); array CGH.

1. Introduction

Comparative genomic hybridization (CGH) is a powerful whole-genome screening technique that allows analysis of DNA copy-number losses (deletions) and gains across the whole genome in a single experiment. Metaphase-CGH, first described by Kallioniemi et al. (1), is based on two-color fluorescence *in situ* suppression hybridization of equal amounts of test (e.g., tumor) and reference (e.g., normal) DNA to karyotypically normal metaphase chromosomes. This is done in the presence of C₀t-1 DNA, which suppresses repetitive elements, allowing them to be excluded from analysis. The smallest deletion that can be detected is approximately 10 Mb, although amplicon units (amplicon size × level of amplification) of approx 2 Mb can be resolved. An overview of the principle of CGH is given in Fig. 1.

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An advantage of CGH is that only genomic DNA (gDNA) is required, allowing screening of formalin-fixed paraffin-embedded (FFPE) tissue archives. Approximately 1 μg of gDNA is required for each experiment, which until recently has necessitated manual dissection of whole tissue sections. As a result very small or focal lesions could not be studied without eliminating the effect of contaminating cells. The combination of laser capture microdissection (LCM), which allows precise dissection under direct visualization, and CGH has now facilitated genomic screening with higher specificity and sensitivity (2–4). Microdissection of as few as five cells (~50 pg DNA) followed by whole-genome amplification (WGA) is sufficient for CGH (3,5–10), but when very small quantities of DNA (<50 pg) are used the genome may be underrepresented, resulting in false CGH profiles (11–13). Ultimately, the appropriate number of cells depends on the method of WGA that is used and also on the nature of the experiment. In our lab we use degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) as the preferential method of WGA.

This chapter describes the procedures involved in performing CGH using DNA probes from laser capture microdissected frozen or FFPE tissue. These include DNA extraction, WGA, DNA labeling, and hybridization. Although the protocols are intended for metaphase CGH, they are also applicable to array-based CGH.

2. Materials

1. Plain noncharged glass slides; absolute ethanol; xylene (flammable, harmful by inhalation and contact with skin); hematoxylin and eosin (H&E) staining reagents; desiccator; and Coplin jars.
2. PixCell II laser capture microdissection system and LCM caps with Capsure transfer film (Arcturus, Mountain View, CA); 0.5-mL Eppendorf tubes, with diameter appropriate for LCM caps, autoclaved/irradiated prior to use.
3. DNA lysis buffer (1X reaction buffer IV [Abgene, Surrey, UK]): 1.5 mM MgCl_2 , 0.45% Nonidet P-40, 0.45% Tween-20; proteinase K, stored at -20°C .
4. Agarose gel electrophoresis equipment, 100 bp and 1 Kb DNA ladder.

Fig. 1. (*opposite page*) Schematic overview of the principle of CGH. Differentially labeled test and normal reference DNA are co-hybridised to normal male target metaphases in the presence of $\text{C}_0\text{t-1}$ DNA. Digital images for the hybridization pattern of test and reference DNA are acquired sequentially using fluorochrome-specific optical filters, and test (green)/reference (red) fluorescence ratio profiles along the axis of each chromosome are determined by computer-based image analysis packages. The method of image analysis shown assigns regions of loss and gain in the test genome on the basis of average test/reference fluorescence ratio profiles crossing predetermined threshold values.

5. DOP-PCR reagents: 6 MW primer 5'-CCG ACT CGA GNN NNN NAT GTG G-3' (MWG Biotech, Milton Keynes, UK); polyethylene glycol ether (PE W1) (Sigma-Aldrich, Dorset, UK); 5X Buffer D (Invitrogen, Paisley, UK); dNTP mix; PCR water; SuperTAQ DNA polymerase (HT Biotechnology, Cambridge, UK); MgCl₂; TAPS buffer (Sigma-Aldrich), all stored at -20°C.
6. PCR machine with ramping facility.
7. CGH nick translation kit (Vysis, Abbott Laboratories, Berkshire, UK), labeled dUTPs stored at -20°C; fluorescent labelled dUTPs, sensitive to light, stored in the dark at -20°C.
8. Deoxyribonuclease (DNase) 1, 10X reaction buffer and stop solution (D5307, Sigma-Aldrich).
9. Sodium acetate.
10. Human C₀t-1 DNA stored at -20°C.
11. Hybrisol VI CGH hybridization buffer (S1370-30 Appligene Oncor) stored in the dark at 2-8°C.
12. Phase contrast microscope.
13. Normal metaphase CGH target slides (Vysis) stored at -20°C.
14. Deionized formamide (toxic and harmful to unborn children), stored in the dark at 4°C.
15. 20X standard saline citrate (SSC) pH 5.3, for denaturation solution.
16. Glass cover slips (22 × 22 mm).
17. Rubber cement (highly flammable).
18. HYBrite denaturing/hybridization system (Vysis).
19. Tween-20.
20. Molecular biology grade.
21. 20X SSC, pH 7.0, for all wash solutions made up fresh with distilled water.
22. Dried milk powder.
23. Fluorescent labeled antibodies, stored in the dark at 4°C.
24. VECTASHIELD® mounting medium with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA), stored in the dark at 4°C.
25. Glass cover slips (22 × 60 mm).
26. Clear nail polish.
27. Fluorescent microscope; image capturing and CGH interpreter software.

3. Methods

The following sections outline the steps that are required for preparation of DNA from both frozen and FFPE tissue for CGH. The experiments are described in the following order: tissue preparation, microdissection, DNA extraction, whole-genome amplification, DNA labeling, hybridization, and analysis. The same process is applied to both the test and reference DNA to be used for each hybridization. We include a description of the two most commonly used DNA labeling protocols (DOP-PCR and nick translation) in **Subheading 3.6.**, although the former is the method of choice in our laboratory. This protocol is also applicable to array-based cases (*see Note 1*).

3.1. Tissue Preparation (see Note 2)

3.1.1. Preparation of FFPE Tissue

1. Cut sections (3.5–4 μm thick) onto plain glass slides.
2. Dewax in xylene, three times for 3 min each, and rehydrate in 100%, 90%, 70% ethanol series and water for 2 min each.
3. Stain with H&E, toluidine blue, or methyl green using standard protocols.
4. Dehydrate in 70%, 90%, and 100% ethanol series and xylene, for 1–2 min each. Leave slides without a cover slip and store at room temperature in a desiccator until required.
5. Immediately prior to use, dehydrate in 100% ethanol, twice for 10 min each, and xylene, twice for 10 min each. Air-dry in a fume hood.

3.1.2. Preparation of Frozen Tissue

1. Snap-freeze tissue and embed in OCT and cut sections at 6–10 μm on a cryostat.
2. Place slides immediately into 70% ethanol in a Coplin jar and leave for 2–4 min to fix. The most important step is the speed at which the slides are transferred to ethanol.
3. Stain slides as in **Subheading 3.1.1., step 3**.
4. Dehydrate in 100% ethanol twice for 10 min each and fresh xylene twice for 10 min.
5. Sections can be used immediately. Alternatively, the slides can be stored in a sealed desiccator at -80°C in batches appropriate for use (e.g., five slides). Slides should be gradually equilibrated to room temperature: at -20°C 24 h prior to use and at 4°C 4–5 h prior to use. Keep at room temperature in a desiccator while setting up the laser capture system.

3.2. Microdissection

We capture a minimum of 50 cells (~500 pg DNA) from test tissue. The reference and test DNA to be cohybridized must be of the same quality. When DNA extracted from FFPE tissue by LCM was cohybridized with DNA from peripheral blood that was diluted and amplified appropriately, we observed inconsistent and false CGH profiles. We use FFPE tissue as a reference for FFPE test tissue. Either LCM or manual dissection can be used to dissect the reference tissue. Capturing is performed according to the manufacturer's instructions for the system that is being used (PixCell II or PALM). We use the PixCell II system. The steps below outline points to be observed for successful capturing.

1. Wear gloves, avoid handling the cap, and use autoclaved/irradiated tubes. It is paramount that all attempts are made to avoid contamination.
2. Keep slides in a desiccator when not in use.
3. Avoid using excessive power (>55 W) for dissection, as it may damage the tissue.
4. Avoid folds in the section and repeated lifting and repositioning of the cap on the section, as the cap must make direct contact with the tissue for successful transfer.

5. Assess the adequacy and purity of each capture by visualizing the cap and residual tissue on the section directly by light microscopy.
6. The dissected material can be left on the cap in an Eppendorf tube at 4°C until required for extraction.
7. If reference DNA is to be obtained from manually dissected tissue, use a clean blade to scrape a 4- μ m section from a plain glass slide prepared as in **Subheadings 3.1.1.** and **3.1.2.** Place the dissected material in a 1.5-mL Eppendorf tube.

3.3. DNA Extraction

Enzymatic digestion is used to extract DNA as follows:

1. Add 0.2–0.25 mg/mL proteinase K to DNA lysis extraction buffer immediately prior to use and add 30–60 μ L buffer to the microdissected tissue. The volume required depends on the yield of microdissection (*see Note 3*).
2. Invert tubes and incubate overnight at 55°C for optimal enzyme activity.
3. Centrifuge tubes at 13,400 ref. for 5 min, replace the LCM caps with a normal PCR tube lid.
4. To inactivate the proteinase K incubate the reaction at 90°C for 10 min, cool on ice for 2 min and centrifuge for 30 s. The extracted material can be stored indefinitely at –20°C.

3.4. DNA Quantitation

1. The amount of DNA to be used for WGA (*see Subheading 3.5.*) and labeling reactions (*see Subheading 3.6.*) must be determined empirically.
2. When small amounts (pg) of DNA are used as a starting template indirect quantitation is used.
3. For primary (1°) DOP-PCR, 5- μ L, 10- μ L, and 15- μ L aliquots of extracted DNA were amplified in parallel reactions and the product visualized on a 2% agarose 1X TBE gel.
4. Ten microliters of extracted DNA from FFPE material (equivalent to approx 10 ng DNA) was found to give optimum results for 1° DOP-PCR (*see Subheading 3.5.2.*).
5. The amount of template to be used for secondary (2°) DOP-PCR will need to be determined in a similar way and will depend on whether it is being used to label the DNA simultaneously (2.5 μ L of 1° DOP-PCR product is found to be adequate) or if nick translation is being to label the DNA subsequent to amplification (12 μ L of 1° DOP-PCR product is required). In either case, this will depend on the quality of the starting material (frozen or FFPE material).
6. We do not quantitate the amount of DNA directly; however, this can be done using the NanoDrop™ ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE).

3.5. Whole-Genome Amplification

DOP-PCR is used to amplify test and reference DNA (*see Note 4*). In the first reaction (1° DOP-PCR), partially degenerate primers are used with initial

low annealing temperatures to prime from multiple sites in the genome. This is followed by a high-stringency amplification step that ensures amplification from sites with the specific primer sequence. A second amplification step (2° DOP-PCR) is used to further amplify DNA and can also be used for labeling. Alternatively, DNA can be labeled by standard nick translation subsequent to 2° DOP-PCR.

3.5.1. Guidelines for All DOP-PCR Reactions

1. The importance of avoiding contamination cannot be emphasized enough. Wear gloves. Prepare all reactions in a PCR hood, ideally with pipets, tubes, water, and pipet tips dedicated to DOP-PCR.
2. Prepare the reaction mixture (number of samples + 0.2) in a PCR hood in a 1.5-mL Eppendorf tube. Add the enzyme last and vortex gently and centrifuge briefly before adding the enzyme.
3. Aliquot reaction mixture into PCR tubes in the PCR hood.
4. Add template outside PCR hood.
5. A negative (PCR water) and positive control (peripheral blood DNA) must be included for all reactions.

3.5.2. Primary DOP-PCR

1. Add the reaction components below in the order listed.

Reagent	Volume (μL)	Final concentration
Water	50-(26+X)	—
5X Buffer D	10	1X
PE W1 (20%)	5	1%
dNTP mix (10 mM)	5	1 mM
6 MW primer (20 mM)	5	2 mM
SuperTaq (5 U/mL)	1	0.1 U/mL
Template DNA ^a	X	—
TOTAL	50	

^a10 μL of extracted DNA from FFPE tissue (*see Subheading 3.4.*)

2. The reaction cycles used are listed below.

Pre-Amplification Step

Temp	Time	Ramp	Number of cycles
94°C	9 min		
94°C	90 s		
30°C	90 s	0.2°C/s-30°C to 72°C	
72°C	180 s		8

(continued)

Amplification step

Temp	Time	Number of cycles
94°C	90 s	
62°C	90 s	
72°C	90 s	27–30

Final extension

Temp	Time	
72°C	480 s	1

3. Visualize the product by running 5-mL on a 2% agarose 1X TBE gel (**Fig. 2**). Highly degraded and small (<50 bp) product should not be used for CGH.

3.5.3. Secondary DOP-PCR

Secondary DOP-PCR amplifies the 1° DOP-PCR at high-stringency conditions. If it is to be used for simultaneous labelling of DNA then proceed directly to the labeling by the 2° DOP-PCR protocol in **Subheading 3.6.1.** (*see Note 5*). Use the following 2° DOP-PCR protocol if nick translation is to be used for DNA labeling.

1. Add the reaction components below in the order listed.

Reagents	Volume (μL)	Final concentration
Water	25–(9.5 + X)	—
10X TAPS Buffer	2.5	1X
dNTP mix (1.6 mM)	2.5	0.16 mM
6 MW primer (20 mM)	2.5	2 μM
MgCl ₂ (25 mM)	1.5	1.5 mM
SuperTaq (5 U/mL)	0.5	0.1 U/mL
Template DNA ^a	X	
TOTAL	25	

^a12 mL 1° DOP-PCR product from FFPE material is required to produce 0.8–1 μg DNA for nick translation; *see Subheading 3.4.*

2. Amplify using the identical high-stringency conditions above (amplification step and final extension in **Subheading 3.5.2., step 2**).
3. Visualize 2° DOP-PCR product by running 2 μL on a 2% agarose 1X TBE gel. Proceed to labeling by nick translation (*see Subheading 3.6.2.*).

Fig. 2. (*opposite page*) Examples of DOP-PCR and labelling. **(A)** Primary DOP-PCR amplified DNA from DNA extracted from laser captured tissue (lanes 3–12). Lane 10 is not suitable for 2° amplification and labeling. The positive (amplified peripheral blood DNA) and negative controls are in lanes 2 and 13, respectively. **(B)**

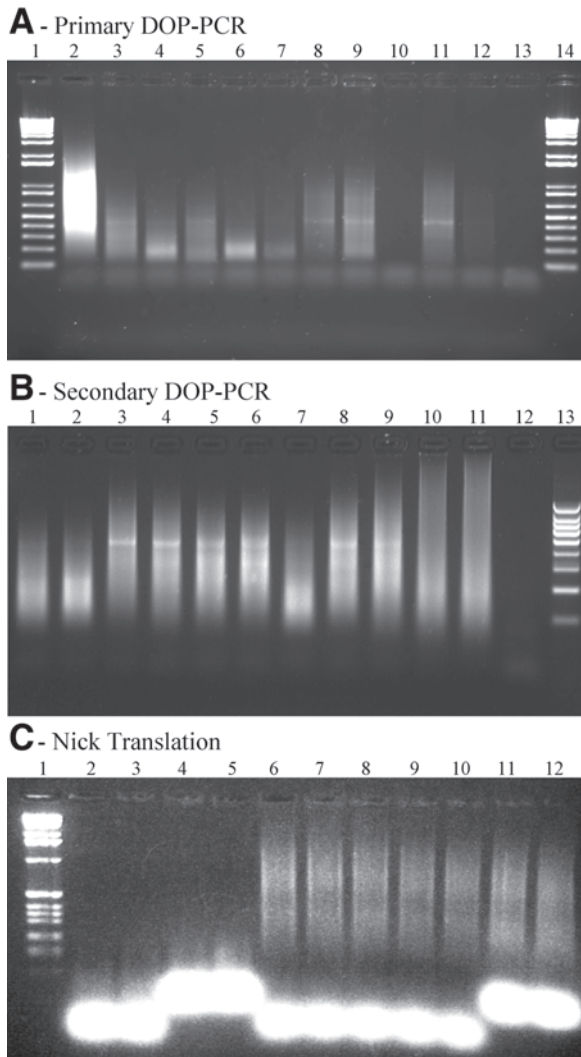


Fig. 2. (*continued*) Secondary DOP-PCR amplified 1° DOP-PCR product. During amplification DNA has been labeled by incorporation of dUTP conjugated to digoxigenin or biotin. Lanes 1–11 contain 2° DOP-PCR amplified product. Samples in lanes 2–9 are suitable for CGH; samples in lanes 10 and 11 need DNase digestion to reduce the probe size to 200–2000 bp. Lane 12 contains the negative control and lane 13 a 100-bp ladder. (C) Secondary DOP-PCR amplified DNA labeled directly with fluorophores by nick translation. Labeled DNA appears as smears 100–1000 bp in lanes 2–12 (1 kb ladder in lane 1). DNA in lanes 6–12 is used for CGH. Labeled DNA in lanes 2–5 are unsuitable for CGH. Unincorporated fluorophores appear as intensely bright signals below the smears.

3.6. DNA Labeling

Adequate DNA labeling is one of the most critical steps in CGH, (*see Note 5*). For CGH, test DNA is always labeled green and reference red either directly, with green (SpectrumGreen-dUTP) and red (SpectrumRed-dUTP) fluorochromes, or indirectly, with digoxigenin-11-dUTP and biotin-16-dUTP. Indirect labeling is more cost-effective and gives a stronger signal but may give increased background fluorescence. The same labeling method should be used for both the test and reference DNA to be cohybridized.

3.6.1. Labeling by DOP-PCR

DNA labeling occurs by substituting labeled dUTP for dTTP along the newly synthesized DNA strand.

1. Add the reaction components as follows:

Reagent	Reaction volume (μL)	Final concentration
Water	25-(8 + X + Y)	—
TAPS buffer (10X)	2.5	1X
dNTP mix ^a	2.5	
6 MW primer (20 μM)	2.5	2 μM
SuperTAQ (5 U/ μL)	0.5	0.05 U/ μL
Template DNA ^b	X	
dUTP-label ^c	Y	
TOTAL	25	—

^adA/C/GTP 2 mM; dTTP 1.6 mM. Final concentration: dA/C/GTP 0.2 mM; dTTP 0.16 mM

^bApproximately 2.5 μL primary DOP-PCR product from FFPE material (*see Subheading 3.4.*).

^c*See Note 6*

2. Amplify using the high stringency amplification step and final extension steps (*see Subheading 3.5.2., step 2*).
3. Proceed to checking the labeled DNA in **Subheading 3.7**.

3.6.2. Labeling by Nick Translation

Nick translation labels DNA using two enzymes, DNA polymerase and DNase 1 (*see Note 7*). Different nick translation kits are available and should be used according to the manufacturer's instructions. In our lab DNA is labeled using the CGH nick translation kit (Vysis) in the following steps:

1. Prepare the reaction mixture (number of samples + 0.2) on ice in a cooled 1.5-mL Eppendorf tube. Mix thoroughly with pipet tip before adding the enzyme. Tap and mix gently with pipet tip after adding the enzyme and centrifuge for a few seconds.

Reagent	Volume (μL)	Final concentration
10X Nick translation buffer	5	1X
0.1 mM dNTP mix	10	0.02 mM
0.1 mM dTTP	5	0.01 mM
Nick translation enzyme	10	

2. Aliquot 30 μL of reaction mixture into 0.2- μL PCR tubes (if using a thermocycler) or 1.5-mL Eppendorf tubes (if using a heat block). Add the following and vortex tube briefly:

Reagent	Volume (μL)
Nuclease-free water	20-(X + Y)
Template DNA ^a	X
Labeled dUTP ^b	Y

^aBetween 8–10 μL secondary DOP-PCR product, equivalent to 0.8–1 μg DNA (see **Subheading 3.4.**)

^bSee **Note 6**

3. Incubate, in the dark if using fluorochromes, for 2–4 h at 15°C.
4. Proceed to checking the labeled DNA (see **Subheading 3.7.**) and leave tube on ice.
5. Stop reaction at 80°C for 10 min, chill on ice, centrifuge.

3.7. Checking the Labeled DNA

Both the quality (probe size) and quantity of labeled test and reference DNA must be checked prior to each hybridization by gel electrophoresis as follows:

1. Run 1/10 volume of labeled DNA on a 2% agarose 1X TBE gel.
2. If probe size is too big (>2000 bp), digest as follows:
 - a. For DOP-labeled DNA use DNase treatment: to 10 μL DNA add: 1 μL DNase 1 (diluted 1:70 with ddH₂O); 4 μL 10X DNase 1 reaction buffer; and 25 μL ddH₂O. Incubate at 15°C for 10–45 min (depending on the initial size of DNA). Add 2 μL stop solution and vortex briefly to stop the reaction. Visualize by running 5 μL on a 2% agarose 1X TBE gel.
 - b. For DNA labeled by nick translation: Add nick translation enzyme (e.g., 1 μL per sample) and incubate further (10 min) prior to stopping the reaction (see **Subheading 3.6.2., step 5.**)

3.8. Probe Precipitation

1. In a 1.5-mL Eppendorf tube, combine equal amounts of labeled test and reference DNA with human C₀t-1 DNA in excess (25–50:1, C₀t-1:probe DNA). For DOP-PCR labeled FFPE probes, 7.5 μL of each probe will suffice and 15 μg C₀t-1 DNA (i.e., 1 μg C₀t-1 DNA/ μL 2° DOP-PCR DNA). If a probe has been digested

as described in **Subheading 3.7., step 2a**, then 30–35 μL of the digested product is equivalent to 7.5 μL of nondigested DNA. If nick translation is used to label frozen DNA, then approx 10–15 μL of each probe is sufficient, depending on the probe quality, with $\text{C}_{0\text{t-1}}$ DNA at 50:1 μg .

2. Add 0.1 volume of 3 M sodium acetate (pH 5.5) and 2.5 volumes of ice-cold 100% ethanol. Vortex tube.
3. Leave at -20°C for at least 3 h or overnight for FFPE tissue.
4. Centrifuge at 13,400 ref for 30 min at 4°C to pellet the DNA.
5. Remove supernatant. Add ice-cold 70% ethanol to remove trace salt and centrifuge at 13,400 ref for 15 min at 4°C .
6. Remove the supernatant. Allow the pellet to dry at 37°C for 2–5 min or 15–20 min at room temperature.
7. Once dry, add 15 μL of hybridization buffer to each tube store at 4°C for at least 30 min or until required.

3.9. Preparation of Metaphase Slides and Probes for Hybridization

Four slides (eight hybridization areas) can be done at one time. Ideally, one control hybridization, normal vs normal (reference DNA labeled red with reference DNA labeled green) should be included with each batch.

1. Leave slides at room temperature for a few hours prior to use.
2. Check the number of metaphases by phase contrast microscopy (5 per 20X field required) and mark a suitable area for hybridization.
3. Take probes from 4°C . Denature at 72°C in a water bath for 10 min, mixing after 3–4 min to ensure the DNA is in solution. Cool on ice for 2 min, briefly centrifuge and incubate in a water bath at 37°C for 30 min to allow the $\text{C}_{0\text{t-1}}$ DNA to hybridize to repetitive elements within the probe DNA.
4. While the probe is denaturing (after 12–15 min), place the slides in prewarmed 50% formamide/50% 2X SSC at 72°C for 2–3 min.
5. Dehydrate slides in 70%, 90%, and 100% ice-cold ethanol series for 3 min each. Dry at 37°C for 1–2 min to evaporate the remaining ethanol.
6. Add 15 μL denatured probe mix to the marked area and immediately apply a glass cover slip (22 \times 22 mm). Seal with rubber cement.
7. Incubate at 37°C for 2–3 d in a humidified dark box or on a HYBrite.

3.10. Washing of Slides and Detecting Hybridization

Following hybridization, the slides are washed in Coplin jars in a shaking water bath, at 42°C unless stated otherwise. Our wash protocol is outlined below but different wash protocols can be used (*see Note 8*). Solutions are prewarmed for 30 min prior to use.

1. Remove cement with forceps. Wash the slides in 50% deionized formamide/50% 2X SSC, three washes for 5 min each to remove excess probe and buffer. The cover slip falls off in the first wash.

2. Wash in 0.1X SSC, three washes for 5 min, to remove nonspecific DNA bound to the chromosomes.
3. Rinse in 4X SST (4X SSC/0.005% Tween-20), three washes for 5 min each. Slides hybridized with directly labeled probes are now ready for mounting (proceed to **step 8**). For indirectly labeled probe, the following steps (**4–7**) are required.
4. Incubate in 5% Marvel in 4X SST for 20 min at 37°C to block nonspecific binding of antibodies.
5. Rinse in 4X SST, for 5 min, three times, then drip-dry.
6. While the slides are still wet, apply 200 μ L fluorescent-labeled antibodies, diluted appropriately in filtered 4X SST, to each slide (*see Note 9*). Cover with a plastic cover slip and incubate for 30 min at 37°C in a humidified chamber in the dark.
7. Remove cover slip and rinse in 4X SST, 5 min, three times.
8. Allow slides to almost dry, drop 20 μ L DAPI with antifade onto slide, cover with 22 \times 60 mm glass cover slip and seal with clear nail varnish.

The slides can be viewed under the fluorescence microscope or stored in the dark at 4°C.

3.11. Visualizing the Hybridization and Analysis

1. Images are captured using a cooled charge-coupled camera linked to a fluorescence Axioplan II microscope (Zeiss, Welwyn Garden City, UK) and VP SmartCapture software (Digital Scientific, Cambridge, UK).
2. The signals are captured independently using three single-band pass filters for tumor DNA (FITC/SpectrumGreen), reference DNA (Cy3/SpectrumRed), and for chromosomes (DAPI).
3. The data from at least 10 images from each hybridization are analyzed by Quips™ CGH/Karyotyper and CGH/Interpreter software (Vysis).
4. For each experiment, a tumor:reference fluorescence ratio (FR) is calculated and compared to a control FR that is obtained from two independently labeled reference DNAs and serves as a reference standard for data analysis.
5. The resulting FR at each locus is equivalent to the ratio of the copy numbers of the corresponding DNA sequences in the test and reference genomes.
6. Therefore, increased green:red FR at a site indicates gain or amplification and reduced ratios represent deletions.
7. The FR change along each metaphase chromosome is quantified. FR values <0.80 and >1.20 are taken to indicate loss and gain, respectively. High-level gain is represented by a FR >1.5 (**I,8**).

4. Notes

1. The protocols in **Subheadings 3.1.–3.5.** for tissue preparation, microdissection, and WGA have also been used to prepare DNA probes of frozen and FFPE material for array-based CGH (AmpliOnc 1, Vysis). For array-based CGH, we labeled DNA using the microarray nick translation kit (Vysis) but alternative labeling methods can be used, e.g., random priming PCR. The labeled product

should, however, be smaller than that required for metaphase CGH, i.e., 200–300 bp instead of 200–2000 bp to hybridize to the target clones on the array. The precipitation, hybridisation, and wash protocols will depend upon the type of array that is used.

2. Use a new blade for each case and do not use adhesive in the water bath. Bake slides briefly (e.g., 10 min) to adhere sections to the slide. If H&E is used, use the minimal concentration that is required to visualize the tissue. Excess stain may interfere with DNA extraction and interpretation of fluorescence (*12,14*). The sections must be completely dehydrated immediately prior to use for efficient dissection: xylene steps can be increased to 30 min or slides can be dried at 30–37°C for 1 h. Dehydration is not a problem for FFPE tissue, but with frozen tissues a balance must be achieved between using increased drying times to dehydrate and the resulting increased adherence to the slide that will prevent capturing.
3. We have rarely required >60 µL extraction buffer when the cap has >50% surface covered (5000 pulses at 30 µm laser diameter). For manually dissected reference tissue, approx 200 µL of buffer is required for DNA extraction. Overnight incubation (14–18 h) is adequate. The caps should be viewed by light microscopy to assess the efficiency of extraction. If undigested material remains after overnight incubation, increase the concentration of proteinase K in the extraction buffer; however, proteinase K at 0.25 mg/mL is usually sufficient. For manually dissected tissue, if tissue is still visible in the extraction buffer after 14–18 h add another 5 µL of proteinase K (20 mg/mL), and incubate further while agitating gently and checking frequently for complete digestion of tissue.
4. DOP-PCR (*15*) requires minimal manipulation of sample DNA and reliably amplifies tiny amounts of DNA to produce probes that are the optimal size for CGH (*2,3,5,6,9,10,16–18*). We modified the original DOP protocol for optimal results with FFPE tissue (*19*). The optimal conditions may need to be altered depending on the tissue being used. We use 6 MW degenerate primers, but multiple specifically designed primers sets can be used to increase genome coverage to 96% (*20*). The same amplification conditions must be applied to the test and reference DNA to be cohybridized. Hybridization of DOP-PCR amplified and labeled test DNA against reference DNA that has been labeled by nick translation but without amplification can show a false CGH profile with deviations at the heterochromatic regions and acrocentric p arms (*4,21*).
5. The choice between 2° DOP-PCR and nick translation for DNA labeling depends on the quality and quantity of the DNA. The former is more suitable for archival material where the DNA fragments are already degraded, as DOP-PCR does not result in a smaller fragment size. If the DNA needs to be digested further an additional partial digestion with DNase 1 can be done after labeling. Nick translation requires 0.8–1 µg of DNA and shortens fragment size to <500 bp because of the action of DNase 1. This amount of DNA can be obtained using the DOP-PCR protocols described in **Subheadings 3.5.2.** and **3.5.3.**; however, we use nick translation only for frozen material where the starting fragment length is 200–2000 bp.

6. Labeled haptens and fluorochromes are used in different concentrations in DOP-PCR and nick translation labeling reactions. The concentration that is used depends on the physiochemical properties of the dye, which affect its incorporation into DNA by DNA polymerase. Different labeling reactions should be performed with increasing concentrations of labeled nucleotide to determine the optimal concentration. Biotin- and digoxigenin-dUTP can be used at high concentrations but efficient labeling takes place when only 1/3 to 1/5 dTTP is replaced. Cy-3- and Cy-5-dUTP are required at much lower concentrations as they have an inhibitory effect at high concentration. We use commercially labeled dUTPs: SpectrumGreen-, SpectrumOrange-, digoxigenin-11-, and biotin-16-dUTPs (Vysis and RocheDiagnostics GmbH, Mannheim, Germany) at 0.01 mM and at 0.04 mM final concentration in our nick translation and DOP-PCR labeling protocols respectively.
7. Nick translation relies on a balance between DNase and DNA polymerase I activity to label DNA. DNase 1 cleaves double-stranded DNA shortening the DNA fragments. The polymerase 1 both removes nucleotides from the 5' end of the nick (5'-3' exonuclease activity) and adds nucleotides to the 3' end (5'-3' polymerase activity). The DNA is labeled when the dTTP in the nucleotide mix is partially replaced with labeled dUTP. The incubation time will vary for the different nick translation kits and will need to be varied for the tissue type (frozen or FFPE tissue). The optimal DNA fragment size for a metaphase CGH probe is 500–2000 bp (**1**) and will be seen for DNA extracted from frozen material; however, DNA from FFPE material is usually 200–1000 bp (**18**). The size of the amplified fragment can be smaller if very small quantities of starting DNA are used as a starting template. Nevertheless, DNA fragments between 200 and 500 bp have yielded successful hybridisations. The single most important factor in assessing probe size is to ensure that the size is equivalent for the test and reference DNA to be cohybridized.
8. Different wash protocols can be used that vary in the concentration of the salt solutions, wash temperature, and wash time. The stringency of the washes can be altered depending on the signal intensity and level of background that is seen. In the protocol described in **Subheading 3.10.**, a salt concentration lower than 2X SSC should not be used with formamide in the first wash in **step 1**, as the resulting higher concentration of the formamide will result in loss of signal. If the loss of signal is too high then lower-stringency washes (0.1–2X SSC) can be used for the second wash in **step 2** but a temperature between 42–45°C is preferable. An alternative quick wash protocol can be used with 0.4X SSC, pH 7.0/0.3% nonidet P-40 at 74 ± 1°C for 2 min followed by 2X SSC, pH 7.0/0.1% nonidet P-40 at room temperature.
9. Fluorescent labeled antibodies must be in solution prior to application by centrifuging at 9300 ref for 10 min prior to use.
If they have precipitated excess granular background may be apparent. We use anti-digoxigenin-FITC (200 µg/mL) and avidin-Cy3 (1 mg/mL) at dilutions of 1:200 and 1:400 respectively in filtered 4X SST.

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