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Comparative Genomic Hybridization: DNA preparation for microarray fabrication

Richard Redon, Diane Rigler, and Nigel P. Carter

Abstract

The spatial resolution of microarray-based comparative genomic hybridization (array-CGH) is dependent on the length and density of target DNA sequences covering the chromosomal region of interest. Here we describe the methods developed at the Wellcome Trust Sanger Institute (Cambridge, UK) to construct microarrays composed of large-insert clones available through genome sequencing projects. These methods are applicable to Bacterial and Phage Artificial Chromosomes (BAC and PAC) as well as fosmid and cosmid clones. The protocols are scalable for the construction of microarrays composed of several hundreds up to several ten thousands clones.

Keywords

microarray fabrication; large-insert clones; BAC; PAC; fosmid; cosmid; DOP-PCR; Comparative Genomic Hybridization; array-CGH.

1. Introduction

Constructing large-insert clone arrays originally involved DNA extraction of DNA inserts from large volumes of bacterial cultures, resulting in only small quantities of DNA available for spotting (1-3). Although this strategy is practical for the construction of small microarrays composed of tens to hundreds of clones, it is not scalable to arrays composed of thousands of clones, such as arrays covering the whole genome with one clone every megabase or with tiling path coverage.

In order to amplify with high fidelity small amounts of large-insert clone DNA obtained from high throughput extraction of small bacterial cultures, several strategies have been developed, such as strand-displacement rolling circle amplification (4) or ligation-mediated PCR amplification (5). Here we describe the protocols for the use of degenerate oligonucleotide-primed PCR (DOP-PCR) (6).

DOP-PCR was originally designed for species-independent general DNA amplification (7). Used with a cycling protocol incorporating a small number of initial low temperature annealing cycles, DOP-PCR allows a general amplification of any target DNA i.e. a chromosomal segment, whole chromosome or whole genome. The target DNA is amplified by PCR with a mix of primers composed of unique 5' and 3' end sequences flanking a random variable hexanucleotide sequence (7).

Although DOP-PCR was designed for general amplification of any DNA fragment, the representation of the target DNA by the DOP-PCR products relies on the six fixed nucleotides located at the 3' end of the primer sequence. In our protocols, each clone is

amplified with three different sets of degenerate primers to improve the representation of large-insert clone sequence after DOP-PCR amplification. Each degenerate primer is designed with a different fixed 3' hexanucleotide sequence, which has been selected for its presence at high frequency in the human genome (6).

In addition, because DNA preparations of human large-insert clones from bacterial cultures are contaminated with *Escherichia coli* genomic DNA (8), the three primer sets have been selected to be inefficient in the amplification of *Escherichia coli* DNA. In consequence, the PCR products using these three primer set contain mostly human insert sequences, which will greatly increase the quantity of printed DNA able to hybridize with the labeled test and reference genomic probes (6).

The three degenerate primers contain the same 5' decanucleotide sequence. This property enables a secondary PCR amplification with one universal 5'-modified primer whose 3' end matches the 5' end of all DOP primers. The secondary PCR presents two advantages: (i) it increases exponentially the quantity of large-insert clone DNA available for array printing; (ii) it facilitates the incorporation of reactive groups for covalent attachment of the amplified DNA to specially coated glass slides. In our protocol, we use 5' amino-linked secondary primers such that amplified large-insert clone DNA will bind covalently to CodeLink® activated slides (6).

2. Materials

2.1. Extraction of large-insert clone DNA

1. 1 ml deep-well microtitre plates (Beckman).
2. 'U' bottom microtitre plate (Greiner).
3. Multiscreen 0.2µM filter plate (Millipore).
4. 2xTY medium: Tryptone 16g/L, Yeast Extract 10g/L, NaCl 5g/L, pH 7.4.
5. Solution I: 50mM glucose, 5mM Tris-HCl pH 8.0, 10mM EDTA, store at 4°C.
6. Solution II: 0.2M NaOH, 1% SDS.
7. Solution III: 3M KOAc, store at 4°C.
8. Isopropanol.
9. 70% Ethanol.
10. T0.1E buffer: 10mM Tris-HCl, 0.1mM EDTA, pH8.
11. RNase A (ICN Biochemicals).

2.2. PCR amplification of large-insert clone DNA

1. Sequences of DOP Primers:
 - (a) DOP1: CCGACTCGAGNNNNNNCTAGAA;
 - (b) DOP2: CCGACTCGAGNNNNNNNTAGGAG;
 - (c) DOP3: CCGACTCGAGNNNNNNNTTCTAG;
 - (d) Aminolink primer: GGAAACAGCCCGACTCGAG.
2. TAPS solution: 250mM TAPS (Sigma), 166mM (NH₄)₂SO₄, 25mM MgCl₂, pH 9.3 (adjusted with 5M KOH). Store at -20°C.

3. TAPS2-buffer: Add 33 μ l of Bovine Serum Albumin (5%) and 7 μ l of β -mercaptoethanol to 960 μ l of TAPS solution. Prepare and sterilize under a UV hood freshly before use.
4. 1% W1: polyethylene glycol ether W-1 (or Brij® 58, Sigma), 1% (w/v) in water.
5. AmpliTaq polymerase (Roche).
6. dNTP mix (each at 2.5mM in water; available from Amersham Biosciences in 100mM solutions).
7. Aminolink buffer: 500mM KCl, 25mM MgCl₂, 50mM Tris-HCl, pH 8.5. Store at room temperature for one week only.

2.3. Array printing

1. CodeLink™ activated slides (GE Healthcare UK Limited, UK).
2. Spotting buffer (4x): 1M sodium phosphate, 0.001% (w/v) sarkosyl, 0.4% (w/v) sodium azide, pH 8.5. Store at room temperature.
3. Ammonium hydroxide: 1% (w/v) in water.
4. 0.1% SDS: Sodium dodecyl sulfate, 0.1% (w/v) in water.

3. Methods

3.1. Extraction of large-insert clone DNA

1. For each clone, set up 500 μ l of bacterial cultures in 2xTY medium with the appropriate antibiotic in a separate well of a 1 ml deep-well microtitre plate. Grow for 18 hours with agitation at 37°C.
2. Transfer 250 μ l of the culture into a 'U' bottom microtitre plate and centrifuge at 1,000g for 4 min when extracting BAC/PAC clones, or for 2 min when extracting cosmids and fosmids.
3. Discard the supernatant, then add 25 μ l of solution I to each well and mix gently by tapping the side of the plate.
4. Add 25 μ l of solution II, mix as before and leave at room temperature for 5 min until the solution clears.
5. Add 25 μ l of cold solution III, mix as before and leave at room temperature for 5 min.
6. After incubation, transfer the content of each well to a 0.2 μ M filter plate. Filter the sample into a 'U' bottom 96-well plate containing 100 μ l of isopropanol per well by centrifugation at 1,000g for 2 min at 20°C.
7. Remove the filter and incubate the samples for 30 min at room temperature before spinning the samples at 1,600g for 20 min at 20°C.
8. Remove the supernatant, wash the DNA pellet in 70% ethanol and centrifuge at 1,600g for 10 min at 20°C.
9. Remove the supernatant, dry the pellet of DNA then leave it to dissolve at 4°C overnight in 5 μ l of T0.1E (pH8) with RNaseA (10 μ l of 1mg/ml RNaseA per 1 ml T0.1E). Store at -20°C until required.

3.2. PCR amplification of large-insert clone DNA

1. For each clone, three separate DOP-PCR reactions are required, with three different DOP primers. For each DOP primer, all reactions are prepared in 96-well plates, each well corresponding to the amplification of one clone in a final reaction volume of 50 μ l (*See Note 1*). The reaction is prepared by mixing: 5 μ l of TAPS2-buffer, 5 μ l of DOP primer (20 μ M); 4 μ l of dNTP mix; 2.5 μ l of 1% W1; 0.5 μ l of AmpliTaq polymerase; 5 μ l of clone template at 1 ng/ μ l (*See Note 2*) and 28 μ l of water.
2. Place the PCR reactions in a thermocycler and use the following program: (i) initial denaturing: 3 min at 95°C; (ii) 10 cycles: 1.5 min at 95°C, 2.5 min at 30°C, ramp up to 72°C by 0.1°C/sec, 3 min at 72°C; (iii) 30 cycles: 1 min at 95°C, 1.5 min at 62°C, 2 min at 72°C; (iv) last extension: 8 min at 72°C
3. Run 5 μ l of each PCR product on a 2.5% agarose gel (200V, 30 min). The average size of the product ranges from 0.2 to 2 kb (Fig. 1A). Ensure you have no signals in the negative controls (*See Note 3*). PCR products can be stored at –20°C until required.
4. Combine together the 3 different DOP-PCR products from each clone into a single 96-well plate position.
5. Prepare aminolink-PCR reactions in 96-well plates, each well corresponding to the amplification of one clone in a final reaction volume of 90 μ l, using the combined DOP-PCR products as a template. The reaction is prepared by mixing: 9 μ l of aminolink buffer, 9 μ l of dNTP mix, 4.5 μ l of aminolink primer (200 ng/ μ l), 0.9 μ l of AmpliTaq polymerase, 3 μ l of combined DOP-PCR products and 63.6 μ l of water.
6. Place the PCR reactions in a thermocycler and amplify using program: (i) initial denaturing: 10 min at 95°C; (ii) 35 cycles: 1 min at 95°C, 1.5 min at 60°C, 7 min at 72°C; (iv) last extension: 10 min at 72°C.
7. Run 2 μ l of the PCR products on a 2.5% agarose gel (200 V, 30 min). The average size of the product ranges from 0.2 to 2 kb (*See Note 3* and Fig. 1B).
8. Transfer 88 μ l of amino-linked DOP-PCR product mix into a Millipore Multiscreen 0.2 μ m filter plate and add 29 μ l 4x spotting buffer (*See Note 4*).
9. Filter the samples by centrifugation at 600g for 10 min and transfer into Genetix 384-well plates. PCR products can be stored at –20°C until required.

¹Because DOP-PCR is a very efficient method for the amplification of template DNA from any organism, it is very sensitive to any trace of contaminating DNA. Always ensure that you are working in a sterile and DNA-free environment, i.e. in a PCR hood. Always work with materials and reagents (water, TAPS2 buffer and 1% W1) that have been UV-treated prior to use; do not UV-treat the AmpliTaq polymerase, dNTPs and primers.

²After DNA extraction, each well should contain approximately 200 ng of DNA dissolved in 5 μ l of T0.1E. Add 195 μ l of water in each well before setting up PCR reactions.

³Negative controls should be included in every PCR set up. For each DOP primer template DNA should be replaced by equivalent volume of water at several positions in 96-well plates to ensure that there is no unexpected DNA contamination. For aminolink-PCR, if combined products from primary DOP-PCR negative controls are used as templates in negative controls, some DNA bands will be detected at corresponding positions after gel electrophoresis (Fig. 1B). However, these DNA fragments are smaller in size than those detected after using DOP-PCR products as templates: their presence is due to the formation of primer oligomers during the first PCR in negative controls, which are then amplified and extended during the secondary PCR reaction. Also include negative controls with only water as template in every secondary PCR set-up and check that there is no DNA product for these reactions.

⁴The 4x spotting buffer is specifically designed to print arrays on CodeLink® activated slides with Microgrid II stations. To print array on slides with another surface chemistry and/or using another printing station, refer to the documentation from the manufacturers.

3.3. Array printing

The protocol in this section was developed at the Microarray Facility of the Sanger Institute (<http://www.sanger.ac.uk/Projects/Microarrays/arraylab/methods.shtml>) where it is routinely used to array DOP-PCR amplified DNA onto CodeLink™ activated slides (GE Healthcare), using Microgrid II stations (Biorobotics). Alternative strategies of microarray printing are described in more details in **Chapters 5-7**.

1. Array DNA elements onto CodeLink™ activated slides at 20-25°C, 40-50% relative humidity.
2. Transfer slides containing arrayed elements into a microscope slide rack, place in a humid chamber containing a saturated NaCl solution and incubate for 24-72 hours at room temperature (*See Note 5*).
3. Remove the slides from humid chamber, block reactive groups by immersing slides in a 1% solution of ammonium hydroxide and incubate for 5 min with gentle shaking.
4. Transfer slides into a solution of 0.1% SDS and incubate for 5 min with gentle shaking.
5. Briefly rinse slides in water at room temperature and then place in 95°C water for 2 min to denature the bound DNA elements.
6. Transfer slides to ice-cold water and then briefly rinse twice in water at room temperature.
7. Dry the slides by spinning in a centrifuge for 10 min at 150g. Store slides at room temperature in a dark and dry place until use (*See Note 6*).

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⁵Long incubations with high humidity levels are used to facilitate covalent binding between the activated surface of the slide and the 5' amino groups of printed DNA elements.

⁶Slides can be stored in the dark under dehumidified atmosphere for at least 3 months without loss of performance in hybridizations. We recommend the use of a sealed desiccation cabinet.

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Figure 1. Banding patterns of DOP-PCR and aminolink-PCR products

(A) Banding patterns of DNA products after PCR amplification of fosmids using DOP1 (top), DOP2 (middle) and DOP3 (bottom) primers. Note the specific banding pattern obtained with each primer. The asterisks (*) mark the absence of any band on negative controls (DOP-PCR without template DNA). (B) Banding pattern of DNA products after aminolink PCR on combined DOP-PCR products. Specific patterns are still visible at this stage. However, patterns tend to be more homogenous with the presence of more bands for each product. Note that all positions corresponding to aminolink-PCR negative controls (*) show the presence of smaller bands: these bands are due to the use of combined products from DOP-PCR negative controls (See Note 2). MW: molecular weight marker.