# *In Situ* Hybridization Protocols

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CHAPTER 5

# Generation of Alphoid DNA Probes for Fluorescence *In Situ* Hybridization (FISH) Using the Polymerase Chain Reaction

***Christoph Lengauer, Ian Dunham, Terry Featherstone, and Thomas Cremer***

## 1. Introduction

Alpha satellite DNA is a primate-specific family of tandemly repeated sequences present in the centromeric regions of all human chromosomes *(1–3)*. The basic unit is a monomer repeat of approx 170 basepair (bp) that contains both sequences conserved among the different chromosomes and variable regions. Blocks of consecutive monomers comprise a chromosome-specific higher-order repeat of up to several kilobases (kb) in size. For most human chromosomes, specific multimeric higher-order repeat units have been described (for review, *see* ref. *4*).

*In situ* hybridization of probes cloned from the *conserved* regions of the alphoid monomer has been applied to human metaphase spreads to pinpoint all centromeric regions simultaneously *(5)*. Such a definition of centromeric regions of all chromosomes may provide marker signals helpful for automated chromosome analysis, in particular in the case of an automated evaluation of radiation-induced dicentric chromosomes *(6)*. In clinical cytogenetics, chromosome-*specific* alphoid probes have become an important aid for the detection of specific numerical chromosome aberrations at all stages of the cell cycle (e.g., *7–11*). In addition, such probes have been exploited in studies of chromosome topography in interphase nuclei and in nuclei of terminally differentiated cells *(12–14)*.

The generation of alphoid DNA probes by cloning procedures is time-consuming and cannot easily be performed in all cytogenetic laboratories. The polymerase chain reaction (PCR) provides a more rapid and easy alternative to generate such probes. Two protocols are described below: The first protocol makes use of oligonucleotide primers for conserved regions of the α-satellite monomer *(15,16)*. In this approach, the specificity of the PCR-generated alphoid probes varies with the source of DNA used for amplification. Using

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human genomic DNA as a source, probes that hybridize to the centromeric regions of all chromosomes can be obtained, while DNA from somatic hybrid cell lines containing single human chromosomes, or DNA prepared from flow-sorted human chromosomes can be used to generate probes for the specific pericentromeric staining of individual chromosomes *(17)*. The second protocol includes oligonucleotide primers for chromosome-specific variable regions of the α-satellite monomer. Using human genomic DNA as a source for PCR, this latter protocol allows the generation of chromosome-specific probes *(17)* (*see* Note 1).

## 2. Materials

### *2.1. PCR with Primers from Conserved Regions of the Alphoid Monomer*

1. Genomic DNA (100 ng) prepared from (a) male human blood, (b) somatic hybrid cell lines containing a certain human chromosome, and (c) libraries of flow-sorted human chromosomes, cloned in the Bluescribe vector (Stratagene, La Jolla, CA) and kindly provided by Joe Gray (University of California, San Francisco).
2. Disposable gloves.
3. Set of microliter pipets (e.g., Gilson).
4. Eppendorf tubes (2.2 mL) with a conical bottom and screw caps.
5. 5X PCR-buffer: 50 m*M* Tris-HCl, pH 8.4, 250 m*M* KCl, 7.5 m*M* $MgCl_2$, 0.005% gelatin. This buffer is stable at –20°C for at least 3 mo.
6. dNTP-mix. Prepare stock solution containing all four dNTPs at a concentration of 1 m*M* from nucleotides purchased from Boehringer Mannheim (GmbH, Mannheim, Germany) (100 m*M* solutions) and store at –20°C for several weeks.
7. *Thermus aquaticus (Taq)* DNA polymerase (Native *Taq*; Perkin-Elmer/Cetus, Norwalk, CT).
8. Oligonucleotide primers α27 and α30 directed to conserved regions located at the 5' end of the human α-satellite monomer *(17):*

   α27: 5' CAT CAC AAA GAA GTT TCT GAG AAT GCT TC 3'

   α30: 5' TGC ATT CAA CTC ACA GAG TTG AAC CTT CC 3'

   Dissolve primers in double distilled water to yield a 100-μ*M* solution each. Mix the two solutions 1:1 to give a final concentration of 50 μ*M* for each primer. Store at –20°C.
9. Salmon sperm DNA sheared or DNase digested to a size of approx 500 bp (Sigma, St. Louis, MO).
10. 20X SSC. 1X SSC is 150 m*M* NaCl, 15 m*M* sodium citrate, pH 7.0.
11. Paraffin oil (light mineral oil).
12. Thermocycler. We use the Thermocycler 60 (BioMed, Theres, Germany) in all our experiments.
13. Seakem ME agarose (FMC Bioproducts, Rockland, ME).
14. 50X TAE: 2*M* Tris-acetate, pH 8.0, 0.05*M* EDTA.
15. 1% Ethidium bromide.
16. DNA gel electrophoresis apparatus.

### *2.2. PCR with Primers from Chromosome-Specific Alphoid Regions*

1. Genomic DNA purified from human blood (500 ng).
2. Disposable gloves.
3. Set of microliter pipets (e.g., Gilson).
4. 0.6-mL Eppendorf tubes.
5. 10X PCR-buffer: 100 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl, 25 m*M* $MgCl_2$, 1.7 mg/mL bovine serum albumin.
6. dNTP-mix. Prepare stock solution containing all four dNTPs (Boehringer Mannheim) at a concentration of 5 m*M* and store at –20°C for several weeks.
7. *Thermus aquaticus* DNA polymerase (Native *Taq*; Perkin-Elmer/Cetus).
8. Oligonucleotide primers directed to chromosome-specific regions of alphoid monomers. The following primer pairs can be used to generate probes specific for the pericentromeric heterochromatin of each of the chromosomes 1, 6, 7, 17, or X (*see* Note 2).

   a. chromosome 1-specific primers *(18)*:

   α1E6: 5' GGC CTA TGG CAG AGG ATA TAA CTG CC 3'

   α1A7: 5' GTG AGT TTT CTC CCG TAT CCA ACG AAA TCC 3'

   The length of the amplification product is 201 bp.

   b. chromosome 6-specific primers *(19)*:

   α6E: 5' ACT GTG GGC TTC AAT GCC GC 3'

   α6F: 5' GCC TAC GGC AGA AAA AGA AAC C 3'

   The length of the amplification product is 182 bp.

   c. chromosome 7-specific primers *(20)*:

   α7A10: 5' TTC ATT GGA ATC GCG AAT AC 3'

   α7A12: 5' CAA GAA GGC TTC AAA GCA CC 3'

   The length of the amplification product is 348 bp.

   d. chromosome 17-specific primers *(18,21)*:

   α17A1: 5' AAT TCG TTG GAA ACG GGA TAA TTT CAG CTG 3'

   α17B2: 5' CTT CTG AGG ATG CTT CTG TCT AGA TGG C 3'

   The length of the amplification product is 227 bp.

   e. chromosome X-specific primers *(22)*:

   αXC11: 5' ATT TCT TTG GAA TCG GGA ATA TTT CCA CAG 3'

   αXD12: 5' CTC TCG TCT TTC TGT GAA GAT AAA G 3'

   The length of the amplification product is 212 bp.

   Dissolve primers in double distilled water to yield 25-μ*M* solution and store at –20°C.
9. Paraffin oil (light mineral oil).
10. Thermocylcer (Perkin-Elmer/Cetus).

## 3. Methods

In this chapter, we have emphasized mainly the procedures for the generation of the alphoid probes and refer the readers to other chapters (e.g., Chapters 4 and 10) in this volume for a more detailed description of the FISH method.

### *3.1. PCR with Primers from Conserved Regions of the Alphoid Monomer*

#### *3.1.1. Performing the PCR*

A PCR carried out in a final volume of 5 µL yields some 1 µg of amplified products sufficent for 50 to 200 FISH experiments. Several identical PCRs are performed simultaneously and amplification products are pooled thereafter.

For each PCR:

1. Prepare the following reaction mix in a 2.2-mL Eppendorf tube:
   a. 1 µL of 5X PCR-buffer;
   b. 1 µL of dNTP-mix (1 m*M* each);
   c. 1 µL of primer-mix (each primer 50 µ*M*); and
   d. 100 ng of a DNA source *(see above).*
   e. If necessary, double distilled water is added to adjust the volume of the reaction mix to 4 µL.
2. Add 1 µL of *Taq* DNA polymerase (2.5 U/µL) and mix gently.
3. Overlay the reaction mix with about 50 µL paraffin oil. This step can be omitted when using the Thermocyler 60 (BioMed).
4. Place the samples in the thermocyler. For the BioMed machine, perform PCR cycles as follows:
   a. 95°C for 3 min;
   b. 95°C for 50 s;
   c. 64°C for 2 min;
   d. 72°C for 3 min;
   e. repeat steps b-d for a total of 30 cycles; and
   f. 72°C for 4 min.
5. Store amplification products at +4°C (up to 1 mo) or at –20°C for more extended periods.

#### *3.1.2. Analyzing the Reactions*

1. Prepare a 100 mL agarose gel (1.2%). Bring the solution to a boil in a microwave oven until all agarose has dissolved. Cool the solution to 55°C, add ethidium bromide (final concentration of 0.5 µg/mL) and pour onto a gel tray.
2. Remove the overlay of oil from the reaction mix. This is done by adding 50 µL of chloroform, mixing and centrifuging for 10 s. The oil dissolves in the $CHCl_3$ (bottom layer). Remove the reaction mix (top layer) with a microliter pipet. Alternatively, use a microliter pipet to directly take up the reaction mix below the oil layer. Remove oil adhering to the pipet tip with a Kimwipe tissue paper. Note that any remaining oil will interfere with the migration of the DNA through the gel.
3. Load a 5-µL aliquot from a pool of several identical PCRs on the gel and electrophorese at 120 V for 1 h.
4. Observe the gel on a UV transilluminator.

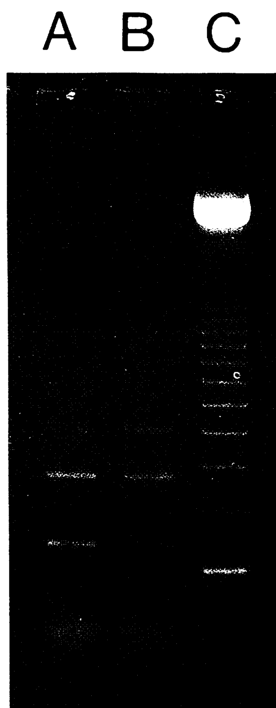



Fig. 1. Agarose gel (1.2%) of alphoid DNA sequences obtained by PCR of DNA of the human–hamster hybrid cell line Cl21 containing the human X chromosome as the only human chromosome material (lane A) and male human genomic DNA (lane B) with the primer pair α27/α30 directed to the alphoid consensus region. Lane C: 123-bp ladder.

An appropriate gel shows a distinct band at approx 170 bp and a ladder of multimers which ends in a smear of up to 3 kb (Fig. 1).

### *3.1.3. Probe Labeling and FISH*

1. Label PCR-products with an appropriate hapten, e.g., biotin-11-dUTP, by nick translation using, for example, the nick translation kit of GIBCO BRL/Life Technologies (Gaithersburg, MD), following the instructions of the supplier.
2. Separate labeled probe from unincorporated nucleotides by standard gel filtration such as using a spin column.
3. Perform fluorescence *in situ* hybridization and washing procedures as described (*23*). Prepare the following hybridization mix (sufficient for one slide using a 24 × 50 mm coverslip):
   a. 5–20 ng of labeled PCR amplified DNA;
   b. 5 µg Salmon sperm DNA;
   c. 5 µL 20X SSC;
   d. Deionized formamide to a final concentration of between 50% and 70% (*see* Note 3); and
   e. Double distilled water to a vol of 50 µL.

4. Add the 50 µL hybridization mixture to a slide with methanol/acetic acid (3:1 v/v) fixed human chromosome spreads.
5. Put a 24 × 50 mm coverslip on the hybridization mixture droplet and seal the edges of the coverslip with rubber cement.
6. For DNA denaturation, transfer the slide into a prewarmed metal box floating in a 73°C waterbath for exactly 10 min.
7. Transfer the box containing the slides to a 42°C waterbath and incubate overnight.
8. Perform washing and detection steps as described in other chapters in this volume.

Some typical results are shown in Figs. 2A–C.

### 3.2. PCR with Primers from Chromosome-Specific Alphoid Regions

#### 3.2.1. Performing the PCR

1. For each PCR, prepare the following reaction mix in a 0.6 mL Eppendorf tube:
   a. 2.5 µL of 10X PCR-buffer;
   b. 1 µL of dNTP-mix (5 m*M* each);
   c. 1 µL of each of the two primers (25 µ*M* each); and
   d. 500 ng of human genomic DNA.
2. Add double distilled water to a vol of 25 µL.
3. Add 1 µL of *Taq* DNA polymerase (1.25 U/µL). Mix gently.
4. Add 25 µL of paraffin oil.
5. Place samples in an automatic thermocyler. For the Perkin-Elmer/Cetus machine, perform PCR cycles as follows:
   a. 94°C, for 2 min;
   b. 94°C, for 1 min;
   c. 65°C, for 2 min;
   d. 72°C, for 2 min;
   e. Repeat steps b–d for a total of 25 cycles; and
   f. 72°C, for 4 min.
6. Store amplification products at +4°C (up to 1 mo) or at –20°C for more extended periods.

#### 3.2.2. Analyzing the Reactions, Probe Labeling, and FISH

Analyze the PCR amplified products by gel electrophoresis and label the probe as described in Sections 3.1.2. and 3.1.3. As an alternative to nick translation, labeled nucleotides, e.g., biotin-11-dUTP, can be incorporated during the PCR using dATP, dCTP, dGTP at a final concentration of 200 µ*M* each, and dTTP as well as biotin-11-dUTP at a final concentration of 100 µ*M* each. *In situ* hybridization, washing, and detection procedures are performed as described *(23)*. An additional washing step with 0.1X SSC at 60°C for 7 min is routinely included. In case of cross-hybridization to the pericentromeric heterochromatin of other chromosomes than the target chromosome the addition of 10 µg/mL unlabeled total human DNA or 1 µg/mL alphoid DNA amplified with the primer pair α27/α30 (*see* Section 2.1., step 8) to the hybridization mixture, and more stringent washing steps can be tried to improve the specificity (Note 3).

Figure 2D shows a normal human lymphocyte metaphase spread after FISH with a probe generated from human genomic DNA with a chromosome 17-specific primer pair.

## 4. Notes

1. The usefulness of the described protocols for PCR-generation of chromosome-specific alphoid probes depends on several factors. A major source of ambiguity which limits the usefulness of alphoid DNA probes for the identification of individual chromosomes stems from the fact that the alphoid sequences of some chromosomes are closely related *(18)*. The construction of oligonucleotide primers for defined subregions of alphoid monomers can be helpful to generate alphoid DNA probes with improved specificity for a given chromosome. The selection of such primers, however, can be difficult and the results are sometimes not predictable in advance.
2. In addition to the primers presented here, primer sets allowing amplification of subsets of α-satellite DNA specific for each of chromosomes 3 and Y have been published by Warburton et al. *(24)*:
   a. chromosome 3-specific primers:

   3-A: 5' TCT GCA AGT GGA TAT TTA AA 3'

   3-B: 5' TGA GTT GAA CAC ACA CGT AC 3'

   The lengths of the amplification products are 1.28 and 0.95 kb.
   b. Y-chromosome-specific primers:

   Y-A: 5' TGA AAA CTA CAC AGA AGC TG 3'

   Y-B: 5' ACA CAT CAC AAA GAA CTA TG 3'

   The length of the amplification product is 1.0 kb.
   For the amplification of alphoid regions specific for chromosomes 7, 17, and X, Warburton et al. *(24)* have described different primer pairs that are also very useful for *in situ* hybridization experiments:
   a. chromosome 7-specific primers:

   7–A: 5' AGC GAT TTG AGG ACA ATT GC 3'

   7–B: 5' CCA CCT GAA AAT GCC ACA GC 3'

   The lengths of the amplification products are 1.0, 0.68, and 0.34 kb.
   b. chromosome 17-specific primers:

   17α1: 5' AAT TTC AGC TGA CTA AAC A 3'

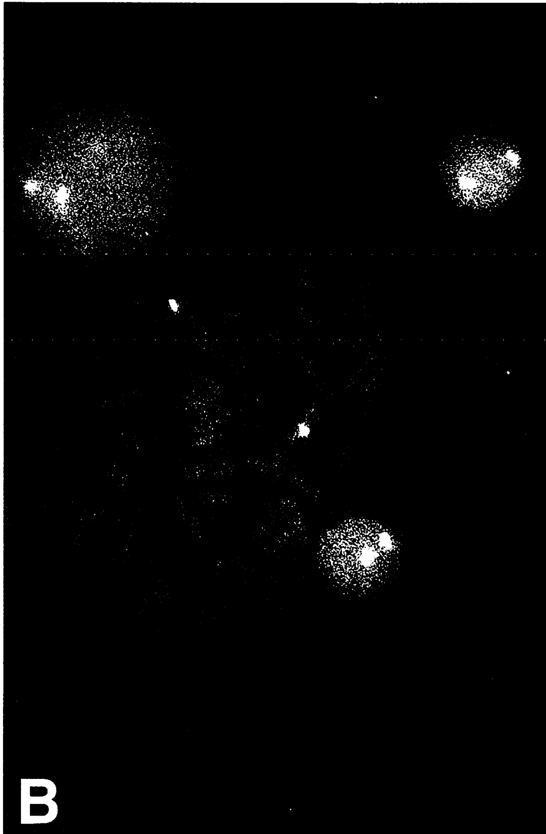
   17α2: 5' TTT AGT TAG GTG CAG TTA T 3'

   The length of the amplification product is 850 bp.
   c. X chromosome-specific primers:

   X-3A: 5' ATA ATT TCC CAT AAC TAA ACA CA 3'

   X-4A: 5' TGT GAA GAT AAA GGA AAA GGC TT 3'

A


B

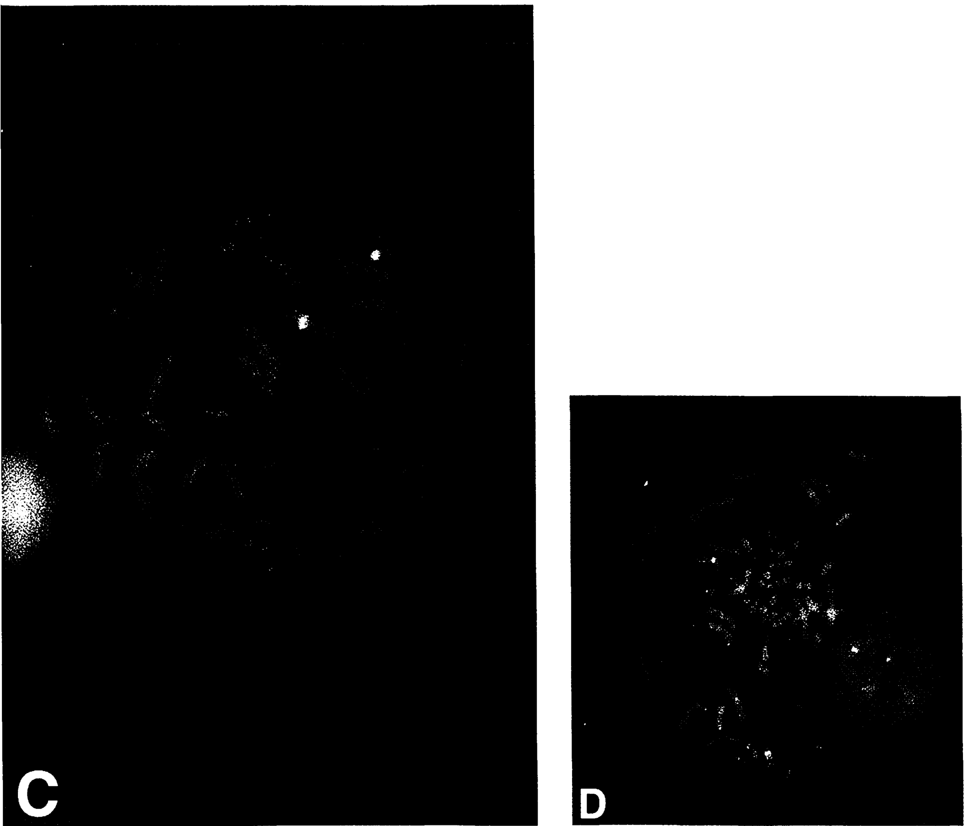



Fig. 2 *(beginning on opposite page)*. FISH of alphoid DNA probes to nomal human lymphocyte metaphase spreads (46,XY or 46,XX). Probes are labeled with biotin-11-dUTP and detected with Avidin-FITC. Chromosomes are counterstained with propidium iodide. (**A–C**) Probes generated by PCR with the oligonucleotide primer pair α27/α30 directed to the alphoid consensus region. (A) FISH using an alphoid probe generated from male human genomic DNA. Note signals on the pericentromeric heterochromatin of all chromosomes of a male metaphase spread. (B) FISH using an alphoid probe generated from the human–hamster hybrid cell line Cl21 containing the human X chromosome as the only human chromosome material. The pericentromeric heterochromatin of the two X chromosomes contained in a female metaphase spread and adjacent interphase nuclei is delineated. (C) FISH using an alphoid probe generated from a plasmid DNA library of flow-sorted human chromosomes 8 selectively decorates the pericentromeric heterochromatin of the respective target chromosomes in a male metaphase spread (*see* Note 4). (**D**) Probe generated by PCR of total genomic human DNA with the chromosome 17-specific oligonucleotide primer pair α17A1/α17B2. Signals are restricted to the pericentromeric heterochromatin of both chromosomes 17.

The length of the amplification product is 850 bp.

3. The stringency of *in situ* hybridization plays a crucial role in avoiding cross-hybridization to other chromosomes *(7)*. For example, the specificity of chromosome-specific PCR-amplified probes critically depends on the formamide concentration, the optimum level of which has to be tested empirically.
4. Note that if chromosome-specific libraries are used as PCR templates, the specificity of probes generated with primers directed to the conserved region of the alphoid satellite monomer may be limited by the presence of alphoid sequences from other chromosomes resulting from impurities during flow-sorting.

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