Extending the Useful Life Span of DNA Probes for Fluorescence In Situ Hybridization

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Whole-chromosome painting probes for fluorescence in situ hybridization (FISH) are comprised of thousands of unique sequences, each of which is represented by numerous copies of the original. These probes are frequently made by using degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) to amplify DNA obtained by microdissection or flow sorting (2,4). Once chromosomes have been obtained by one of these methods, they are subjected to 30 or 40 cycles of PCR. This product becomes the original stock solution, and an aliquot is then labeled with a reporter molecule (e.g., biotin or rhodamine) and used as a painting probe for FISH. When this probe is gone, more is made from the unlabeled PCR products. To avoid the necessity of flow sorting or microdissecting again, an aliquot of the original stock is re-amplified without a label, providing a new stock of unlabeled DNA from which probe can be made.

Such probes have a finite useful life span, which is partially determined by the number of PCR cycles through which the probe has been taken. Each time the stock DNA is replenished by PCR, the resulting product tends to have less complexity than the precursor. A complex probe will contain enough specific sequences to paint a chromosome evenly; stochastic losses of individual DNA sequences tend to result in a probe that yields uneven labeling and hence shows a banded pattern. The primary reason for the loss of...
complexity is probably unequal amplification because of failure to form a template-primer match or failure of the polymerase to replicate the template DNA completely. Unequal numbers of copies in early rounds leads to much larger relative differences following exponential expansion. Successive rounds of PCR exacerbate this problem until the stock product is no longer suitable for making labeled probes.

We undertook to determine the number of cycles of PCR that could be performed on a DNA template before probes adequate for chromosome painting could no longer be produced. This subject is of interest to any laboratory that makes and uses a large quantity of FISH probes. We chose to test two different commercially available DNA polymerases: Thermo Sequenase® (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and AmpliTaq® DNA Polymerase (PE Biosystems, Foster City, CA, USA). AmpliTaq is a “standard” Taq DNA polymerase and is commonly used in PCRs. Thermo Sequenase, while less commonly used as a polymerase for PCR, has a very high processivity that we thought might improve the ability of the polymerase to fully copy the template strand. The DOP-PCR using AmpliTaq had been optimized for magnesium concentration and pH in our lab before these tests, and the reaction conditions used here were the result of the optimization process. The Thermo Sequenase reaction conditions were those specified by the manufacturer. In each case, the number of polymerase units used was also specified by the manufacturer’s instructions. However, since the recommended number of units per 50 µL reaction is 20 for Thermo Sequenase and 3 for AmpliTaq, we also performed reactions with 20 U of AmpliTaq to see if there was a discernible difference based solely on the quantity of polymerase used.

We dissected single copies of human chromosomes on which DOP-PCR in situ had been performed before microdissection; a process that we developed and refer to as HeadStart Microdissection (1). These chromosomes were amplified by DOP-PCR in 15-µL reactions that contained 20 U Thermo Sequenase DNA Polymerase, 26 mM Tris-HCl, pH 9.0, 6.5 mM MgCl₂, 200 µM of each dATP, dGTP, dCTP and dGTP (Boehringer Mannheim, Indianapolis, IN, USA) and 4 µM DOP primer (5′-CCGACTCGAGNNNNNNATGGTG-3′). Mineral oil (30 µL) was added to the reaction mixture to prevent evaporation. The thermal profile consisted of (i) 95°C for 10 min, (ii) 6 cycles at 94°C for 1 min, 30°C for 2 min, (iii) a ramp of 0.1°C/s up to 65°C for 3 min, (iv) 30 cycles at 94°C for 1 min, 57°C for 1 min and (v) 72°C for 3 min, followed by 72°C for 5 min. Reactions were held at 4°C until removed from the thermal cycler. A 2-µL aliquot from this reaction, considered first generation, was used as a template for the subsequent generation. Subsequent PCRs were performed, each using 2 µL of products from the previous generation as a template in a 100-µL reaction volume identical in makeup to that described above, until six generations were created. To verify that DNA amplification had occurred, each sample was visualized on a 1.5% agarose gel at 100 V for 1 h. Once all generations were created, painting probes were made from each by performing an additional 30 cycles of PCR in which a fluorescent dye was incorporated. A 50-µL reaction solution identical to that described above was used, but was supplemented with 40 µM tetramethylrhodamine-6-dUTP (the concentration of dTTP was unchanged). The thermal profile consisted of 95°C for 5 min, 25 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 3 min followed by 72°C for 5 min.
for 5 min, and PCR products were incubated at 4°C until removed from the thermal cycler.

The painting probe was added to a hybridization mixture (50% formamide, 2× standard saline citrate [SSC], 10% dextran sulfate and 3 µg of human COT-1 DNA® [Life Technologies, Gaithersburg, MD, USA]) to a final volume of 15 µL. Microscope slides containing human metaphase spreads from lymphocytes were denatured in 70% formamide (in 2× SSC, pH 7.0) followed by a dehydration series in 70%, 85% and 100% ethanol solutions for 3 min each. The probe was denatured at 70°C for 5 min and applied to the slides, which were then covered with 22-× 22-mm coverslips, sealed with rubber cement and hybridized for 2 days at 37°C.

Unbound probe was removed with three 5-min washes in 50% formamide, 2× SSC, pH 7.0 (45°C), followed by one 5-min wash in 2× SSC (45°C) and one 5-min wash in 2× SSC with 1% Triton® X-100 (45°C). The metaphase chromosomes were then counterstained with 4′,6-diamidine-2-phenylindole (DAPI) and an anti-fade solution. Metaphase spreads were observed using an Axioskop® Fluorescence Microscope (Carl Zeiss, Thornwood, NY, USA) with a dual-band-pass filter.

Figure 3. Photographs of chromosome paints that were generated using either Thermo Sequenase (Panels A, B and C) or AmpliTaq (Panels D, E and F). Panels A and D show second-generation products, Panels B and E show third-generation products, and Panels C and F show fourth-generation products. All photos were taken with a Quips system, using single-band-pass excitation filters for DAPI and Rhodamine and a triple-band-pass emission filter.
with AmpliTaq have lost sequences during PCR amplification, resulting in less complexity. Interestingly, we saw no difference between reactions run with 3 U of AmpliTaq vs. those run with 20 U. For purposes of comparison, all AmpliTaq data shown were obtained from the reactions run with 3 U (the recommended amount).

The functionality of each probe was determined visually, using a dual-band-pass filter (DAPI and TRITC) and a triple band-pass emission filter (DAPI/TRITC/FITC).

Amplification products from each test were electrophoresed on a 1.5% agarose gel to determine if any difference in the size of the PCR products could be detected. DNA amplified using Thermo Sequenase (Figure 1A) was significantly larger than that amplified with AmpliTaq (Figure 2B). This could indicate that the probes made with AmpliTaq have lost sequences.

REFERENCES


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