GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Primed *in situ* labeling for detecting single-copy genes

J.-L. Gao[1,2], Y. Nie[1] and X.-P. Ding[1]

[1]Key Laboratory of Bio-Resources and Eco-Environment, Institute of Medical Genetics, College of Life Science, Sichuan University, Ministry of Education, Chengdu, China
[2]ChengDu Blood Center, Chengdu, China

Corresponding author: X.-P. Ding
E-mail: brainding@scu.edu.cn

Genet. Mol. Res. 10 (3): 1884-1890 (2011)
Received March 18, 2011
Accepted July 4, 2011
Published August 26, 2011
DOI http://dx.doi.org/10.4238/vol10-3gmr1391

ABSTRACT. In order to analyze male sterility caused by deletion of SRY and DAZ, we examined the accuracy and cost-effectiveness of a modified primed *in situ* labeling (PRINS) technique for detection of single-copy genes. Peripheral blood samples were collected from 50 healthy men; medium-term cultured lymphocytes from these samples were suspended in fixative solution and then spread on clean slides. We used four primers homologous to unique regions of the SRY and DAZ regions of the human Y-chromosome and incorporated reagents to increase polymerase specificity and to enhance the hybridization signal. PRINS of SRY and DAZ gave bands at Yp11.3 and Yq11.2, respectively, in all 50 metaphase spreads. The PRINS SRY signals were as distinct as those obtained using traditional fluorescence *in situ* hybridization (FISH). This new method is ideal for rapid localization of single-copy genes or small DNA segments, making PRINS a cost-effective alternative to FISH. Further enhancement of PRINS to increase its speed of implementation may lead to its wide use in the field of medical genetics.

Key words: PRINS; FISH; Single-copy gene; SRY; DAZ

INTRODUCTION

Primed *in situ* labeling (Pellestor et al., 1995; Tharapel and Wachtel, 2006) is a method of target DNA sequence detection and localization, which combines features of fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR), and was developed as a chromosome labeling technique based on repetitive alpha satellite sequences. The primed *in situ* labeling (PRINS) technique uses oligonucleotide primers that are annealed and extended on chromosome preparations on microscope slides in the presence of labeled nucleotides. After extension with Taq DNA polymerase, the relevant sequences are visualized by fluorescence microscopy (Koch et al., 1989). With the vast DNA sequence information made available through the Human Genome Project, it is likely that PRINS primers could be developed to detect single-copy genes with chromosomal localization as an alternative to FISH. Detection of SRY and DAZ is important in the study of sperm development and Y-chromosome formation (Chandley et al., 1989; Habermann et al., 1998), and therefore, we aimed to test the effectiveness of the PRINS method in this capacity by attempting to detect two different Y-specific sequences that are involved in genetic disease. The test determines that the SRY gene and the azoospermia factor (AZF) subregion DAZ are involved with 46,XY gonadal dysgenesis and male infertility, respectively (Wachtel, 1998; Kent-First et al., 1999; Hargreave, 2000). We used PRINS primers specific for these Y-chromosome regions, and modified the technique to allow greater hybridization specificity and signal strength. We also compared the accuracy and efficiency of the modified PRINS technique with traditional FISH.

MATERIAL AND METHODS

Sample collection and preparation

Informed consent was obtained and peripheral blood samples were collected from 50 healthy men. Standard cytogenetic techniques were used to obtain lymphocytes from medium-term cultures. Metaphase spreads were prepared by suspending lymphocytes in fixative solution (3:1, methanol:glacial acetic acid) and then spreading on clean slides. The slides were successively dehydrated in 70, 85, and 100% ethanol baths for 2 min, and then air dried for PRINS or FISH.

Primers, PRINS, and FISH

We used four primers (Invitrogen Corporation, China) specific for the SRY gene (Kadandale et al., 2000) (5'-GCAGGGCAAGTAGTCAACGTT-3', 5'-AAGCGACCCATGAACG CATTC-3', 5'-AGAAGTGAGCCTGCCTATGTT-3', 5'-GCCGACTACCCAGATTATGGA-3') and four primers specific for DAZ (Kadandale et al., 2002) (5'-CTCTGCCTCTGGCTTTAC CA-3', 5'-GAGGAGGCATCTGGAAATCATT-3', 5'-GGAAGCTGCTTTGGTAGATAC-3', 5'-TAGGTTTCAGTGTTTGGATTCCG-3'). A Blast search of the primer sequences showed that they were specific for their intended targets (Figure 1).

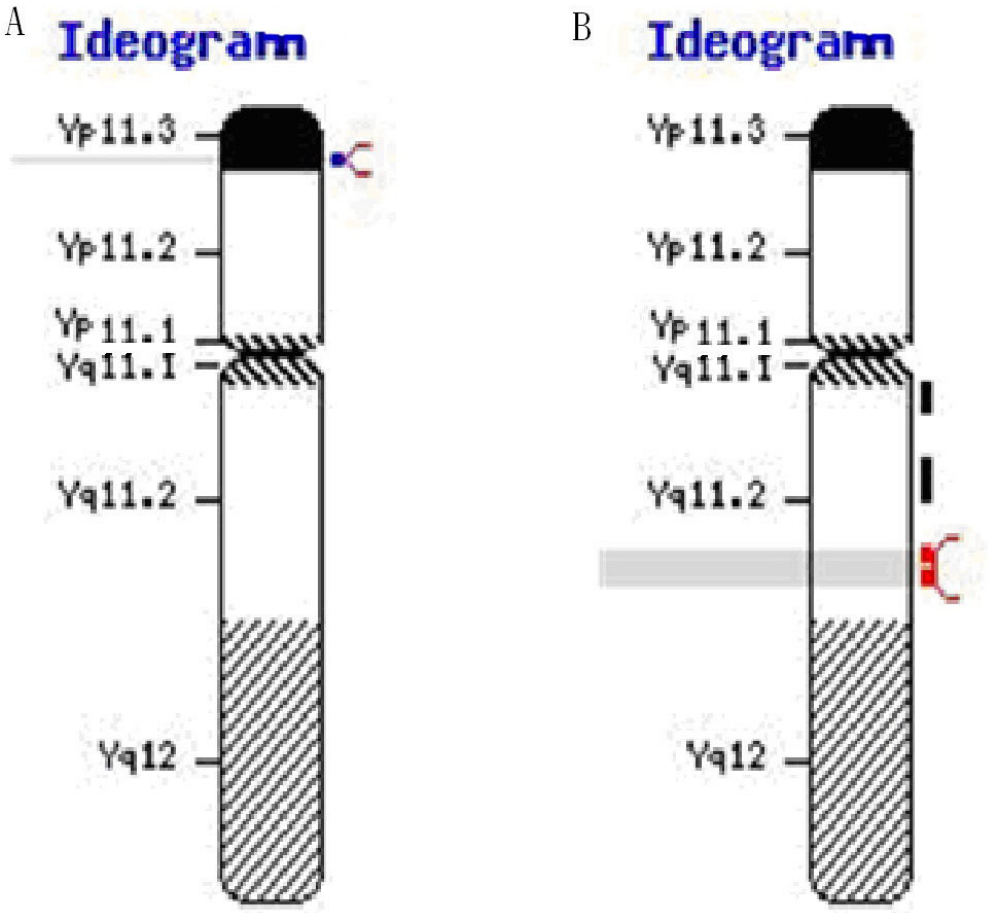

Figure 1. A. SRY and **B.** DAZprimers' blast results.

DNA denaturation of the metaphase spreads prepared from peripheral blood was carried out by incubating the slides in 70% deionized formamide and 2X SSC at 72°C for 5 min, followed by successive passes through ice-cold ethanol baths (70, 85, and 100%) for 2 min each, and then air-dried. The 25-µL PRINS reaction mixture [100 pmol each primer, 0.2 mM dNTPs (N = A/C/G; Promega Corporation, USA), 0.02 mM dTTP, 0.02 mM biotin-16-dUTP (Roche Applied Science, USA), 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$, 0.01% BSA, 1 U Taq DNA polymerase, and 0.44 µg TaqStart antibody (Takara Bio Inc., Japan)] was prepared and heated to 60°C. A 12.5-µL volume of reaction mixture was added to the denatured metaphase spread slide, and then covered with a coverslip. The slide was placed on a PCR block (Peltier Thermal Cycler MG48G, LongGene Scientific Instruments, China) and incubated at 55°C for 5 min to allow primers to anneal, and then at 72°C for 10 min to allow primer extension. The coverslip was then removed, and slide was placed in 0.4X SSC, 0.3% NP-40 (AMRESCO Inc., USA) at 72°C for 2 min and shaken for 3 min to remove unbound primers and unincorporated dNTPs.

Enhancement of the signal was carried out using the Tyramide Signal Amplification (TSA; Biotin System, PerkinElmer, Waltham, MA, USA). Before the slide was completely dry, 12.5 µL 20 µg/mL avidin-rhodamine (Roche Applied Science) was added and incubated at 37.5°C for 30 min. The slide was washed three times for 5 min in TNT buffer (from the TSA kit) at 37.5°C and then air dried. Seven microliters 0.06 µg/mL DAPI Antifade (Sigma Corporation, USA) was added to the slide, incubated at room temperature for 10 min, and then air dried.

FISH detection of the SRY gene was performed using the Vysis SRY FISH probe (Abbott Co. Ltd., USA) according to manufacturer instructions.

Signal detection and image analysis for PRINS

In order to reduce errors in detection, two detection systems were used to observe the fluorescence signals: the Axioplan 2 imaging microscope system (Carl Zeiss Inc., Germany) equipped with Zeiss Plan-NEOFLUAR 100/1.30 oil objective lens, and the Olympus BX51 fluorescence microscope (Olympus Corporation, Japan) equipped with Olympus UPlanFI 100 X/1.30 oil ∞/0.17 C1 objective lens. Both systems have DAPI/FITC/rhodamine band-pass filters and all slides were examined by two independent observers. The photomicrographs were taken using an AxioCam camera module and a COHU Cooled CCD Camera module, and then analyzed using ISIS 5.0 (MetaSystems Company, Germany) and VideoTest-FISH 4.0 softwares (VideoTesT Ltd., Russia), respectively.

For each detection system, at least 50 metaphase spreads of a sample were scored for distinct colored signals. The sizes and intensities of signals were compared, and two signals were distinguished if they were separated by at least the diameter of the smaller nucleus. Overlapping chromosome spreads or nuclei without a well-defined boundary were not scored.

RESULTS

We collected blood from 50 healthy male volunteers, and 50 metaphase spreads were prepared from each volunteer. Using a modified PRINS method, we were able to detect SRY and DAZ sequences with the same accuracy as standard FISH. In all metaphase spreads subjected to PRINS for SRY, we observed a red fluorescent signal at position Yp11.3, which was comparable to the signal obtained using standard FISH (Figure 2). Furthermore, the signal intensity of PRINS was the same as that obtained using FISH. We also used this PRINS technique to detect DAZ and observed a clear red fluorescent signal at Yq11.2 (Figure 3).

DISCUSSION

PRINS was developed as a chromosome labeling technique to detect aneuploidy. The traditional PRINS protocol utilized primers specific for repetitive alpha satellite sequences (Kallioniemi et al., 1992; Pellestor et al., 1996; Pellestor, 2006). Because of the large number of copies available for hybridization, interference with hybridization by chromatin or other proteins would still allow a strong enough signal to be visualized. In order to detect single-copy genes using PRINS, modifications must be made to the protocol to allow for efficient and accurate labeling (Koch et al., 1995; Yan et al., 2001).

The first consideration is the number of primers to use. A single primer would not al-

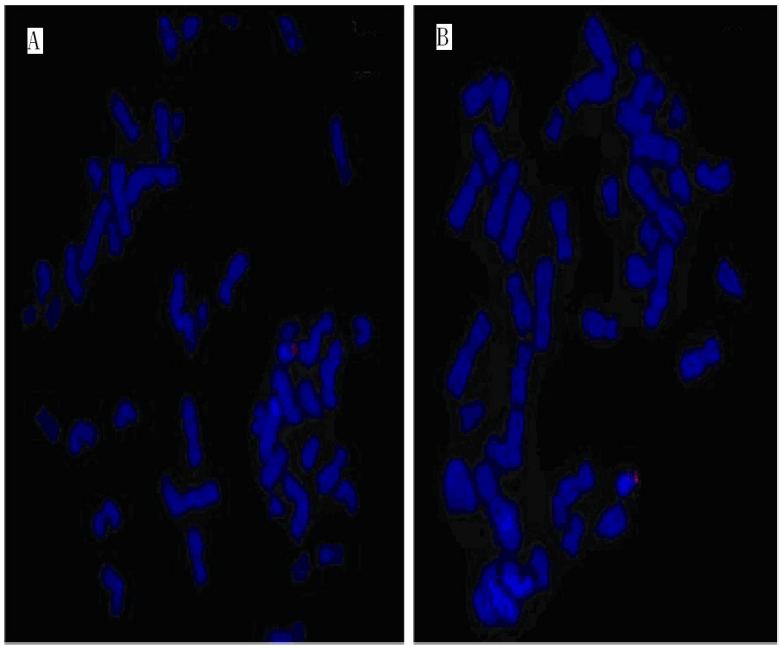

Figure 2. PRINS-labeled SRY gene results compared to FISH. **A.** Four SRY primers using the PRINS technique. **B.** Labeled SRY gene using the Vysis SRY FISH probe. A and B, red signal position is Yp11.3.

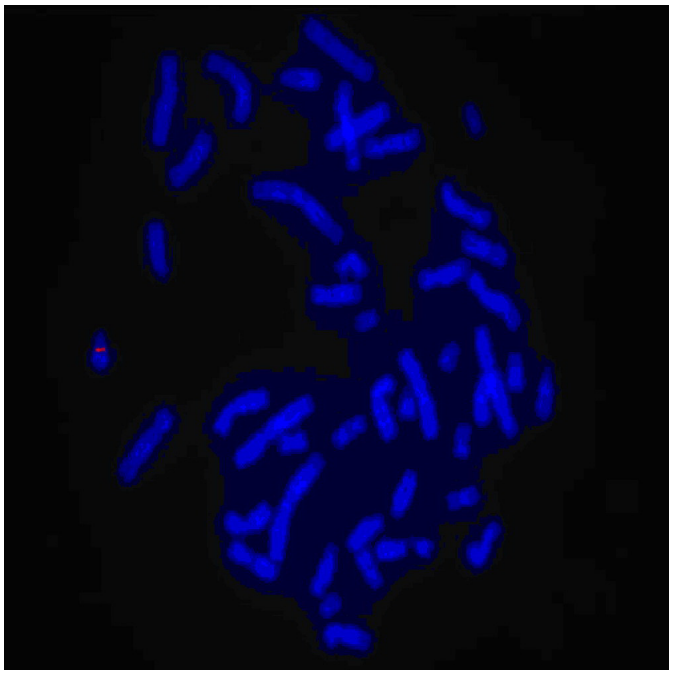

Figure 3. PRINS-labeled DAZ gene result. Red signal position is Yq11.2.

low a strong enough signal for fluorescent detection. Too many primers would likely lead to primer-dimers and/or non-specific hybridization. We used four primers specific for the SRY region and four primers specific for DAZ (Figure 1).

In order to improve the polymerase specificity, we used TaqStart, a neutralizing monoclonal antibody to Taq DNA polymerase, which prevents non-specific amplification and formation of primer-dimers. TaqStart antibody is used to block polymerase activity during setup of the PCRs at ambient temperatures (20° to 22°C). The inhibition of Taq DNA polymerase is completely reversed when the temperature is raised above 70°C.

To aid detection of the SRY and DAZ sequences in the metaphase spreads, we used a commercially available kit to enhance the fluorescent signals. In the TSA Biotin System, the biotin-labeled dUTP that is incorporated into the primer extension products becomes bound by streptavidin-HRP conjugate. Biotinyl Tyramide then reacts with HRP. Fluorescence visualization is possible by the use of an avidin-fluorophore conjugate. We employed stringent washing in 0.4X SSC and 0.3% NP-40 to remove unbound primer to achieve minimal background.

We improved the techniques previously published (Koch et al., 1989; Tharapel and Wachtel, 2006) in the following ways. First, by using 0.4X SSC, 0.3% NP-40, wash times were shorter. Moreover, a minimal background was obtained for easy signal detection. Second, a smaller volume of PRINS reaction mixture was used, reducing the cost of the PRINS reaction. Finally, we reduced the time needed for primer extension to 10 min, increasing the efficiency of the PRINS reaction.

We obtained identical results with PRINS and FISH for the detection of SRY in metaphase spreads. Others have reported similar results in the study of the dystrophin gene (Cinti et al., 2002). While the laboratory time required for PRINS detection was the same time as FISH, the PRINS process costs much less in terms of reagents (Velagelati et al., 1998; Tharapel and Kadandale, 2002; Pellestor et al., 2002; Zeng et al., 2006). Therefore, as an alternative to FISH, our modifications to the PRINS technique should allow for the chromosomal localization of single-copy sequences, and the identification of certain rearrangements. Using this technique, we can determine whether SRY or DAZ is missing. If SRY or DAZ is undetected, there will be no signal on specific loci of the chromosome. Consequently, a preliminary conclusion can be drawn to assist further clinical diagnosis. In addition, we believe that this modified PRINS technique can have very meaningful applications in molecular cytogenetics. It can be used for the visualization and mapping of genetic loci on chromosomes, and for detection of the presence or absence of small DNA segments involved in genetic diseases. PRINS can be a cost-effective alternative to FISH. Further modifications of the technique may lead to increased time efficiency over that of FISH.

REFERENCES

Chandley AC, Gosden JR, Hargreave TB, Spowart G, et al. (1989). Deleted Yq in the sterile son of a man with a satellited Y chromosome (Yqs). *J. Med. Genet.* 26: 145-153.

Cinti C, Stuppia L and Maraldi NM (2002). Combined use of PRINS and FISH in the study of the dystrophin gene. *Am. J. Med. Genet.* 107: 115-118.

Habermann B, Mi HF, Edelmann A, Bohring C, et al. (1998). DAZ (Deleted in AZoospermia) genes encode proteins located in human late spermatids and in sperm tails. *Hum. Reprod.* 13: 363-369.

Hargreave TB (2000). Genetic basis of male fertility. *Br. Med. Bull.* 56: 650-671.

Kadandale JS, Wachtel SS, Tunca Y, Wilroy RS Jr, et al. (2000). Localization of SRY by primed *in situ* labeling in XX

and XY sex reversal. *Am. J. Med. Genet.* 95: 71-74.
Kadandale JS, Wachtel SS, Tunca Y, Martens PR, et al. (2002). Deletion of RBM and DAZ in azoospermia: evaluation by PRINS. *Am. J. Med. Genet.* 107: 105-108.
Kallioniemi A, Kallioniemi O-P, Waldman FM, Chen LC, et al. (1992). Detection of retinoblastoma gene copy number in metaphase chromosomes and interphase nuclei by fluorescence *in situ* hybridization. *Cytogenet. Cell Genet*. 60: 190-193.
Kent-First M, Muallem A, Shultz J, Pryor J, et al. (1999). Defining regions of the Y-chromosome responsible for male infertility and identification of a fourth AZF region (AZFd) by Y-chromosome microdeletion detection. *Mol. Reprod. Dev.* 53: 27-41.
Koch JE, Kolvraa S, Petersen KB, Gregersen N, et al. (1989). Oligonucleotide-priming methods for the chromosome-specific labelling of alpha satellite DNA *in situ*. *Chromosoma* 98: 259-265.
Koch JE, Hindkjaer J, Kolvraa S and Bolund L (1995). Construction of a panel of chromosome-specific oligonucleotide probes (PRINS-primers) useful for the identification of individual human chromosomes *in situ*. *Cytogenet. Cell Genet.* 71: 142-147.
Pellestor F (2006). Development and adaptation of the PRINS technology: an overview. *Methods Mol. Biol.* 334: 211-220.
Pellestor F, Girardet A, Lefort G, Andreo B, et al. (1995). PRINS as a method for rapid chromosomal labeling on human spermatozoa. *Mol. Reprod. Dev.* 40: 333-337.
Pellestor F, Girardet A, Lefort G, Andreo B, et al. (1996). Rapid chromosome detection in human gametes, zygotes, and preimplantation embryos using the PRINS technique. *J. Assist. Reprod. Genet.* 13: 675-680.
Pellestor F, Imbert I and Andreo B (2002). Rapid chromosome detection by PRINS in human sperm. *Am. J. Med. Genet.* 107: 109-114.
Tharapel AT and Wachtel SS (2006). PRINS for mapping single-copy genes. *Methods Mol. Biol.* 338: 59-67.
Tharapel SA and Kadandale JS (2002). Primed *in situ* labeling (PRINS) for evaluation of gene deletions in cancer. *Am. J. Med. Genet.* 107: 123-126.
Velagelati GV, Shulman LP, Phillips OP, Tharapel SA, et al. (1998). Primed *in situ* labeling for rapid prenatal diagnosis. *Am. J. Obstet. Gynecol.* 178: 1313-1320.
Wachtel SS (1998). X-linked sex-reversing genes. *Cytogenet. Cell Genet.* 80: 222-225.
Yan J, Bronsard M and Drouin R (2001). Creating a new color by omission of 3 end blocking step for simultaneous detection of different chromosomes in multi-PRINS technique. *Chromosoma* 109: 565-570.
Zeng M, Ding X, Jiang M, Li X (2006). Rapid chromosome detection by PRINS in human sperm. *Reprod. Contracept.* 26: 241-244.

