THE PRINS TECHNIQUE AND ITS APPLICATIONS IN HUMAN CYTOGENETICS

LA TÉCNICA PRINS Y SUS APLICACIONES EN CITOGENÉTICA HUMANA

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SUMMARY

The advent of molecular genetic techniques has brought forth new procedures for *in situ* chromosomal analysis. One of these techniques is PRINS (primed *in situ* labelling), a fast and sensitive technique that uses unlabeled DNA primers. I discuss this the history AND evolution of this technique and its various components.

RESUMEN

Con el desarrollo de nuevas técnicas en genética molecular se han producido nuevas tecnologías para el análisis cromosómico mediante hibridacion *in situ*. Una de estas metodologías es el PRINS (Marcaje in *situ* mediante *primers*) que es rápido y sensible y utiliza *primers* no marcados de ADN. En este trabajo se discute la historia y evolución de esta técnica y sus componentes.

The advent of molecular genetic techniques has brought forth new procedures for *in situ* chromosomal screening and aneuploidy detection. Fluorescence *in situ* hybridization (FISH) technique has become the method of choice for *in situ* chromosomal detection because its allows the direct *in situ* vizualization of chromosome copy numbers. The method has found numerous applications in human cytogenetics. In the majority of these studies, the probes used are centromeric repeat a-satellite probes because the corresponding fluorescent signals are bright and easily scorable. However, the use of a-satellite probes is hampered by the lack of specificity of these probes for some chromosomes, especially acrocentrics which share a high homology in their a-satellite DNA sequences, resulting in cross-hybridization in FISH reactions (Willard and Waye, 1987; Lebo *et al.*, 1992).

A new alternative for *in situ* chromosomal screening is the primed *in situ* (PRINS) labeling technique. This method combines the high sensitivity of the PCR reaction with the cytological localization of specific DNA sequences.

In PRINS procedure, chromosomal

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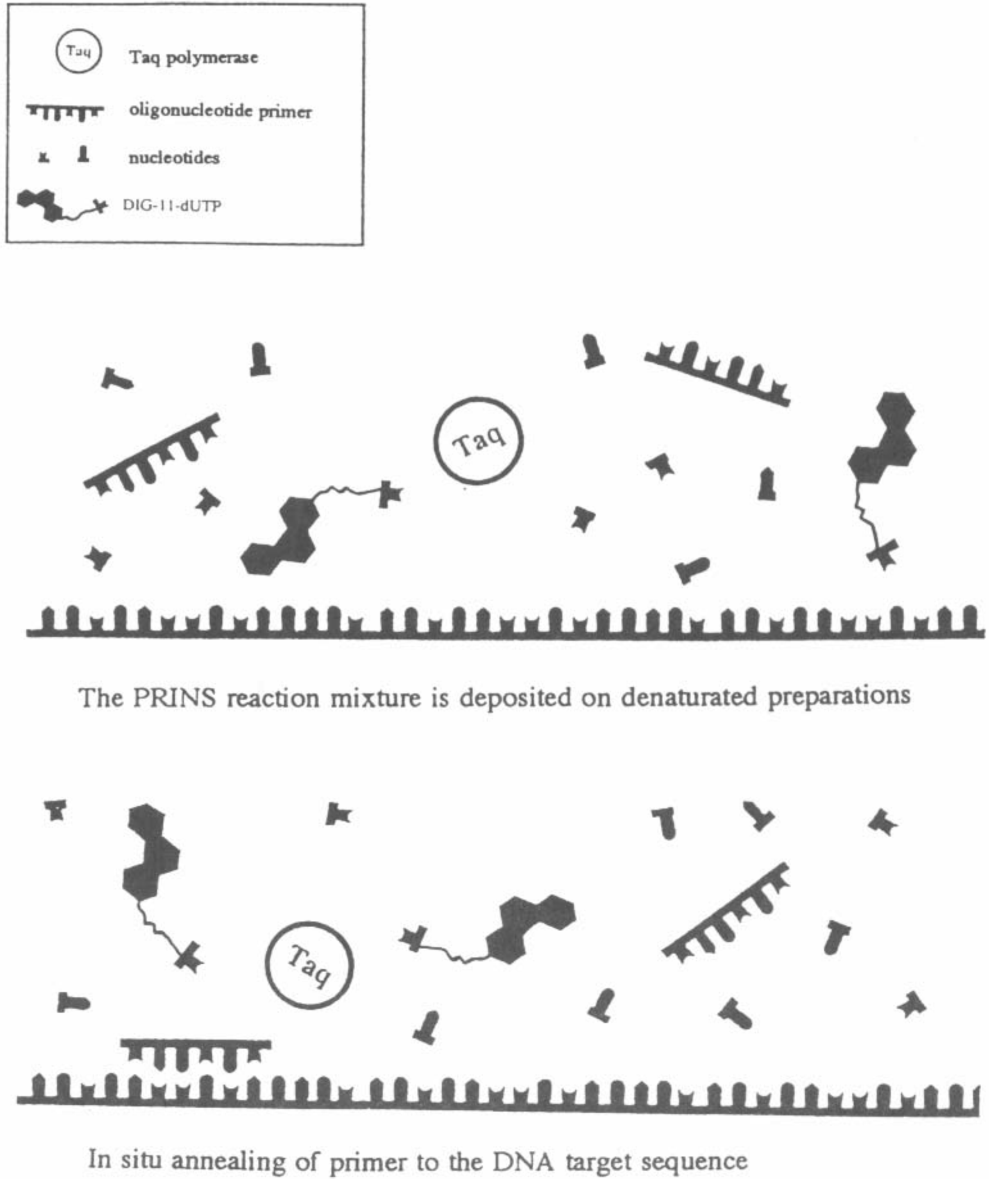

Figure 1. *Principles of primed* in situ *labeling reaction. After annealing of oligonucleotide primers to the DNA template, the primer extension is performed by a Taq DNA polymerase in the presence of free nucleotides of which at least one is labeled. Thus, the site where the chain elongation occurs is labeled and can be visualized using fluorescence microscopy either directly if fluorochrome-labeled nucleotide is used, or indirectly after detection by protein conjugated with fluorescent reporter molecules.*

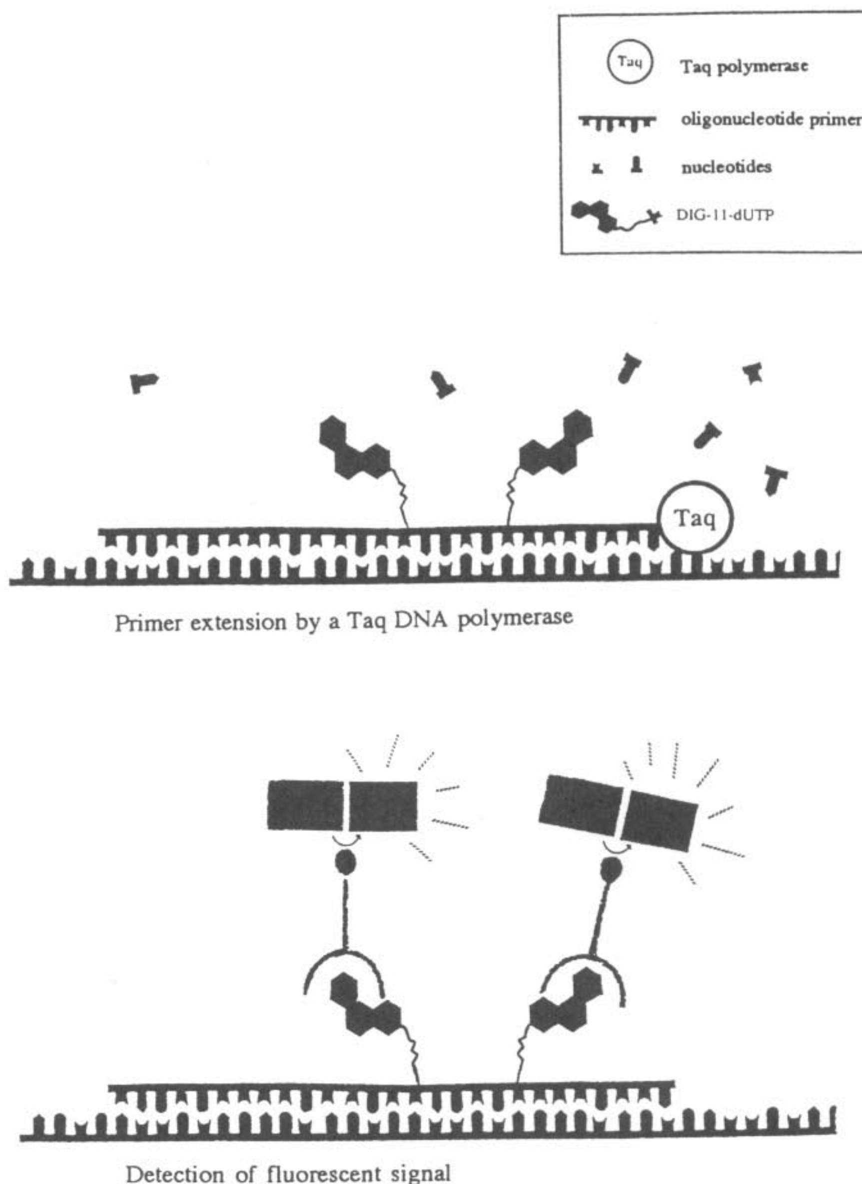

Figura 1. Principios de la reacción de marcaje *in situ*. Después del templado de los cebadores del oligonucleótido, la extensión del cebador es realizada mediante una Taq polimerasa en presencia de nucleótidos libres, de los cuales, al menos uno, está marcado. De este modo, el lugar donde se produce la elongación de la cadena queda marcado y puede ser visualizado usando microscopía de fluorescencia directamente, si se emplea nucleótido marcado con fluorocromo, o indirectamente, después de la detección mediante proteína conjugada con moléculas informadoras fluorescentes.

identification is performed by *in situ* annealing of specific and unlabeled oligonucleotide primers to complementary sites on denatured chromosome spreads or nuclei. The annealed primers provide initiation sites for chain elongation, catalyzed by a Taq DNA polymerase in the presence of free nucleotides of which at least one is labeled (Koch *et al.*, 1989). The principles of PRINS are outlined in **figure 1**.

Cells or tissue samples are fixed and denatured before PRINS reaction in order to both preserve morphology and permit access of the reagents to the sequence target. The reaction may be performed eitheron intact cells in suspension or in cell preparations or tissue sections on glass slides.

Labeling of human chromosomes has been obtained using oligonucleotide primers for repeated DNA sequences. Various repeat families are spread over the human genome and involve satellites, Alu or telomeric DNA sequences. An advantage of primers is their ability to differentiate between closely related sequences. This feature has been utilized for generating chromosome-specific primers from the alpha-satellite DNA motif. These centromeric repeats are made up of a variable number of monomeres of 171 bp in length and are organized as alpha-satellite subfamilies. The DNA sequences of monomeres slighly deviate among subfamilies and individual chromosomes. The chromosome specificity of PRINS labeling is then based on the use of primers generated from these chromosome-specific alpha-satellite DNA sequences. The complementation process between the primer and its centromeric target will be so specific that a simple mismatch between the 3'-end of the primer and the genomic sequence will prevent initiation of the elongation by the Taq DNA polymerase. Thus, it has been possible to define specific alpha-satellite primers for some chromosomes undistinguishable by FISH with centromeric probes (Koch *et al.*, 1995; Pellestor *et al.*, 1995), such as chromosomes 13 and 21 which share 99.7 p. cent homology in theiralpha-satellite DNA sequences. The high discriminating power of PRINS has been demonstrated in a study on polymorphism in alpha-satellite sequences from chromosomes 13 (Pellestor *et al.*, 1994).

In pratice, specific alpha-satellite primers were defined by comparing the alpha-satellite sequence of each chromosome to the consensus alpha-satellite sequence established by Choo *et al.* (1991). The primer sequences were determined in the area with the most nucleotide divergence. Using an automatic DNA synthesizer, both the preparation and the purification of oligonucleotides are now fast and cheap. The length of primers ranges from 18 to 35 nucleotides. Their small sizes facilitate their accessibility to the genomic sequences. To date, specific primers have been defined for 20 human chromosomes. Their specificity has been successfully tested on both metaphases and interphase nuclei. As an alternative to oligonucleotides, cloned probes fragmented by restriction enzyme digestion can also be used as primers. Because they are unlabeled, high amounts of primers can be used in PRINS reaction without background problems. This

makes the PRINS a very fast technique.

Initially, PRINS reactions were performed either in a thermoblock or a waterbath. The weakness of these methods was in the lack of stringency of primer annealing. In fact, these procedures did not allow precise and durable temperature control. The procedure has been considerably improved by using a programmable temperature cycler equipped with a flat plate block. With this equipment, the precision of temperature control may reach 0.1°C and the required temperature changes are both easy to program and rapidly carried out. The use of an automatic thermocycler allows an optimization of both annealing and extension conditions.

For each PRINS reaction, a mix is prepared in a final volume of 50 µl containing the oligonucleotide (50 to 250 pM), the nucleotide mixture including a labeled dUTP (biotine, digoxigenine, fluoresceine, coumarine or rhodamine), the Taq polymerase buffer and 1-2 units of enzyme. The mix is preheated on a waterbath at the annealing temperature of the used primer. The denatured preparation slide are put on the plate block of thermocycler. The reaction consists of 2 programmed steps: 10 minutes at the specific annealing temperature of the used primer and 5-30 minutes at 72°C in order to allow the nucleotide chain elongation.

The slide is not sealed. Both the volume of the mix and the short incubation time prevent the slide from drying during the reaction. The PRINS reaction is stopped bt immersing the slide in a stop buffer (500mM NaCl, 50mM EDTA, pH 8) at 72°C for 1 minute, and the slide is transferred in 2xSSC, 0.5 p. cent Tween 20 at room temperaure. In case of multi-target PRINS reaction, the slide is then treated 15 minutes at 37°C with a dideoxynucleotides mix and 2 units of Klenow enzyme in order to block the free 3'-ends of the elongation fragments generated by the first PRINS reaction. This intermediate step prevents mixing of labeling. The slide is washed and again placed on the plate block of the thermocycler. A second PRINS reaction mix, involving a primer specific for another chromosome and another labeled dUTP, is applied to the slide and a new reaction is run. A second blocking reaction may eventually be performed and a third PRINS reaction can be run. Detection of the labeling sites is performed by immunocytochemistry and conventional fluorescence microscopy. A simple target PRINS reation can be performed in 15 minutes, using directly labeled nucleotides. In sequential PRINS reactions, 3 distinct chromosomes can be identified in a less than 2.30 hour reaction.

A number of applications of PRINS have already been successfully developed in humans, mammaliansor plants, demonstrating that PRINS method could be easily adapted to various types of cells.

In human cytogenetics, various applications of PRINS method have been successfully tested such as assessment of aneuploidy in lymphocytes, amniocytes and preimplantation embryos (Brandt *et al.,* 1993; Pellestor *et al.*, 1996a). Recent studies have presented the use of PRINS for the direct estimation of disomy rates in human sperm (Pellestor *et al.*, 1996b). The PRINS

methodology was combined with an efficient 3M NaOH treatment allowing the simultaneous decondensation and denaturation of sperm nuclei. The technique has also been applied to chromosomal screening of somatic hybrid, tumor cell lines and fetal cells in maternal blood.

These recent achievements with PRINS procedure show that this new molecular technique has the potential to constitute an efficient complement to PCR and FISH. The PRINS technique combines two essential features required for cytogenetic diagnosis, i.e. rapidity and specificity. On the other hand, the availability of primers specific for chromosomes 13, 16, 18, 21, X and Y is of the utmost interest for clinical application since these chromosomes are the most frequently involved in human aneuploidy.

PRINS has emerged as a research technique. With the development of rapid and simplified protocols producing reliable and reproductible results, this technique has an enormous potential for cytogenetic diagnosis.

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