

A Simplified Combination of DNA Probe Preparation and Fluorescence *in situ* Hybridization

Dino Celeda^{a,b} Ulrich Bettag^a, Christoph Cremer^a

^a Institut für Angewandte Physik, Universität Heidelberg,
D-W-6900 Heidelberg, Bundesrepublik Deutschland

^b Institut für Humangenetik und Anthropologie, Universität Heidelberg,
D-W-6900 Heidelberg, Bundesrepublik Deutschland

Z. Naturforsch. **47c**, 739–747 (1992); received May 14, 1992

Fluorescence *in situ* Hybridization, DNA Probe Preparation, Simultaneous Labeling, Modified Nick Translation, PCR and PCR Buffers, Human Lymphocytes, Digoxigenin

Fluorescence *in situ* hybridization (FISH) has found widespread applications in cytogenetics. So far the standard protocols for probe amplification (and simultaneous labeling) by PCR, nick translation and *in situ* hybridization involve different buffer systems leading to a number of time consuming washing steps even before hybridization. In this manuscript we show a fast technique of a close combination of DNA probe preparation and *in situ* hybridization (ISH).

This method was applied to metaphase chromosomes from human lymphocytes fixed on slides. Two specific repetitive DNA probes, the pUC 1.77 DNA probe and the DYZ1 repetitive DNA fraction were used, amplified and labeled in different ways. Additional experiments with total genomic male human DNA as the DNA probe suggest that this method may be extended to a large variety of other probes. Moreover the ISH technique described does not require toxic denaturing agents, such as formamide.

Introduction

The method of non-isotopic (especially fluorescence) *in situ* hybridization (FISH) has become an important adjunct in cytogenetics including prenatal chromosome diagnostics, cell biology, tumor cytogenetics, biological dosimetry, chromosome evolution and gene mapping [1–10]. Up to now reliable successful protocols for FISH are available for a large number of specific DNA probes even as small as some hundred base pairs [10]. In principle these FISH techniques are transferable to FISH in suspension with slight suspension specific modifications [11].

DNA probe amplification by polymerase chain reaction (PCR) has become an important technique also for FISH [12–14, 17]. In this context it has been shown that amplification and simultaneous labeling of specific DNA sequences, for example with hapten modified nucleotides is possible [14]. In combination with suitable parameters (e.g. primers, time intervals of heating/cooling and buffer systems) the technique of PCR is a fast alternative for the preparation of DNA probes. Starting

with a small fraction of the DNA template, a large amount of even already labeled DNA probe can be obtained within a few hours.

Here we present an *in situ* hybridization (ISH) method based on a close combination with DNA probe preparation. This was realized by the use of PCR buffer systems for: amplification of the DNA probe by PCR; simultaneous labeling or labeling by nick translation after PCR; ISH (PCR buffer plus SSC).

Materials and Methods

Chromosome preparation

Chromosomes were obtained from human lymphocytes isolated from peripheral blood by standard techniques [20]. Lymphocytes were cultivated for 72 h followed by a Colcemid block of 3 h. Metaphase chromosomes were prepared according to the hexandiol method [11] and fixed on slides by means of methanol/acetic acid (3:1).

Buffers

Three buffers were used for DNA probe preparation and (fluorescence) *in situ* hybridization ((F)ISH):

1) Buffer (a), 10 ×: Tris-HCl 100 mmol/l, MgCl₂ 30 mmol/l, KCl 500 mmol/l, gelatine 100 µg/ml,

Reprint requests to Prof. C. Cremer.

Verlag der Zeitschrift für Naturforschung,
D-W-7400 Tübingen
0939–5075/92/0900–0739 \$ 01.30/0



Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung“) beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

This work has been digitized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition "no derivative works"). This is to allow reuse in the area of future scientific usage.

pH 8.3 (20 °C). This buffer was used for DNA probe preparation (amplification, simultaneous labeling, and labeling by modified nick translation) and *in situ* hybridization of pUC 1.77 [21, 26] and DYZ 1-repetitive DNA fraction [17].

2) Buffer (b), 5 × [17]: 50 mmol/l Tris-HCl, pH 8.0 (Carl Roth GmbH, Karlsruhe, F.R.G.), 15 mmol/l MgCl₂ (J. T. Baker Chemicals B.V., Deventer, Netherlands); 0.25 vol% Tween 20 (Serva, Heidelberg, F.R.G.); 0.25 vol% octyl-phenol-ethylene oxide condensate (Sigma Chemical Company, St. Louis, Mo., U.S.A.). This buffer was used for amplification, labeling by modified nick translation with digoxigenin-11-dUTP (DIG-11-dUTP) and ISH of pUC 1.77.

3) Buffer (c), 10 ×: Tris-HCl 100 mmol/l, MgCl₂ 15 mmol/l, KCl 500 mmol/l, gelatine 100 µg/ml, pH 8.3 (20 °C). This buffer was used for amplification and simultaneous labeling with digoxigenin-11-dUTP (DIG-11-dUTP) by PCR and ISH of the DYZ 1-repetitive DNA fraction.

Amplification and labeling of DNA probe pUC 1.77 (see Table I)

All PCRs were performed in a device built in our laboratory [14]. The DNA probe pUC 1.77 designates a clone of the plasmid vector pUC 9 containing a 1.77 kb long human *Eco* RI fragment as insert, which was isolated from the human satellite DNA fraction II/III. This insert mainly represents a tandemly organized repetitive sequence in the region q12 of chromosome # 1 [21], with minor binding sites on other human chromosomes [16].

In buffer (a) this DNA probe was generated and labeled in three different ways:

pUC probe (1), amplified by PCR and labeled with DIG-11-dUTP by modified nick translation: The entire plasmid vector pUC 9, containing a 1.77 kb long human *Eco* RI fragment as insert, was used as target DNA: 7.8 ng; primers (Reverse, Sequencing; MWG-Biotech, Ebersberg, F.R.G.):

Table I. Table of the DNA probes (probe preparation) used for ISH in different buffer systems. Characters (a)–(c) indicate the different buffers used (see Materials and Methods). Numbers in brackets indicate different preparation methods as described in Materials and Methods.

Probe preparations for probe preparation and ISH	Buffer		
Amplification by PCR and:	buffer (a)	buffer (b)	buffer (c)
labeled by modified nick translation DIG-11-dUTP	pUC probe (1)	pUC probe (4)	
labeled by simultaneous incorporation DIG-11-dUTP	pUC probe (2)		DYZ 1 probe (2)
labeled by simultaneous incorporation DIG-11-dUTP and DNase treatment	DYZ 1 probe (1)		
pUC probe (3)			
Probe preparation	ISH in buffer (b)		
No amplification by PCR labeled by standard nick translation DIG-11-dUTP in standard buffer system	human genomic male DNA*		
Probe preparation	ISH in buffer with formamide		
Total plasmid DNA labeled by nick translation DIG-11-dUTP	pUC probe (5)		

* Isolation of DNA from human lymphocytes in aqueous solution; ISH was performed in buffer (b).

50 ng each; dNTPs (Boehringer Mannheim, Mannheim, F.R.G.); 20 nmol each; Taq-polymerase (Boehringer Mannheim, Mannheim, F.R.G.); 5 units; amplification buffer: buffer (a), 5 µl; H₂O (Milli-Q ZFMQ 23044, Millipore, Eschborn, F.R.G.); to make up a final volume of 50 µl. The reaction volume was overlayed with Bayol-F paraffin oil (Serva, Heidelberg, F.R.G.); 30 µl. PCR was performed within 40 cycles: annealing: 1.1 min, 64 °C; extension: 4.0 min, 75 °C; denaturation: 2.0 min, 94 °C.

After PCR, 1.7 nmol DIG-11-dUTP and 2 µl of enzyme mix (Boehringer Mannheim, Mannheim, F.R.G.) were added to 20 µl of the reaction volume. Incubation for 90 min at 19 °C. The nick translation was stopped by incubating the volume for 5 min at 94 °C.

pUC probe (2), amplified and simultaneously labeled with DIG-11-dUTP by PCR: Amplification with simultaneous labeling by PCR was done according to the *pUC probe (1)* amplification protocol (see above) with the following modifications: nucleotides: 18 nmol dTTP and 2 nmol DIG-11-dUTP instead of 20 nmol dTTP; Taq: 10 units; annealing: 1.1 min, 60 °C; extension: 6.0 min, 75 °C.

pUC probe (3), amplified and simultaneously labeled with DIG-11-dUTP by PCR and treated with DNase: After PCR, prior to ISH to an aliquot of 10 µl (approx. 800 ng of amplified DNA) of the reaction volume 0.8 units DNase 1 (Boehringer Mannheim, Mannheim, F.R.G.) were added and the volume was then incubated for 90 min at 19 °C. Reaction was stopped by incubation for 5 min at 94 °C.

In buffer (b) the *pUC 1.77* DNA probe was generated and labeled in the following way:

pUC probe (4), amplified by PCR and labeled with DIG-11-dUTP by nick translation: For amplification by PCR the entire plasmid was taken, containing the human DNA insert of 1.77 kb's according to the preparation protocol described elsewhere [14] with the exception that no hapten modified nucleotides were used, and following additional modifications: The amplification was carried out by running 35 cycles: 1.5 min at 45 °C (annealing), 4.0 min at 72 °C (extension), 1 min at 92.0 °C (denaturation), each.

After PCR, to an aliquot of 25 µl (approx. 700 ng of amplified DNA) of the volume, 10 µl

(4 units) DNase/polymerase 1 mix (GIBCO BRL, Eggenstein-Leopoldshafen, F.R.G.) and 2 nmol DIG-11-dUTP (Boehringer Mannheim, Mannheim, F.R.G.) was added and the volume was then incubated for 90 min at 16 °C. Reaction was stopped by the addition of 6 µl of 3 mmol/l EDTA (GIBCO BRL, Eggenstein-Leopoldshafen, F.R.G.).

Amplification and labeling of DYZ1 DNA probe (see Table I)

As a second DNA probe the DYZ1-repetitive DNA fraction (main binding site in the heterochromatin of the Y chromosome) was used. Amplification was carried out by using sequencing and reverse sequencing primers [17], and total human male DNA. The DNA probe was labeled simultaneously by PCR according to the following protocols:

DYZ1 probe (1), amplified and labeled in buffer (a): Template DNA (total genomic male human DNA): 2.3 ng; buffer (a) 10 ×: 5 µl; primers WYR 007, WYR 008 (27 mers): 210 ng each; dATP, dGTP, dCTP: 20 nmol each; dTTP: 12 nmol; DIG-11-dUTP: 8 nmol (all nucleotides, Boehringer Mannheim, Mannheim, F.R.G.); Taq-polymerase (Boehringer Mannheim, Mannheim, F.R.G.): 10 units; addition of distilled water (Milli-Q ZFMQ 23044, Millipore, Eschborn, F.R.G.) to make up a final volume of 50 µl; in Safe-Lock 0.5 ml Eppendorf reaction tubes (Eppendorf-Netheler-Hinz GmbH, Hamburg, F.R.G.) the mixture was overlayed with Bayol-F paraffin oil 30 µl (Serva, Heidelberg, F.R.G.).

Prior to PCR the reaction volume (without Taq-polymerase) was incubated for 5 min at 91 °C and immediately put on ice for additionally 5 min.

After addition of Taq-polymerase to the reaction volume, PCR was performed within 40 cycles: annealing: 4.0 min, 61 °C; extension: 3.5 min, 75 °C; denaturation: 3.0 min, 94 °C.

DYZ1 probe (2), amplified and labeled in buffer (c): Amplification and labeling were done according to the preparation protocol DYZ1 probe (see above) with the following modifications: buffer (c); annealing: 4.0 min, 57 °C; extension: 3.5 min, 70 °C; denaturation: 3.0 min, 94 °C.

Labeling of total genomic human male DNA with DIG-11-dUTP by nick translation in buffer (b) (see Table I)

Total genomic human male DNA was labeled by nick translation (Boehringer Mannheim, Mannheim, F.R.G.) according to product informations.

In situ hybridization (see Table I)

ISH was performed on human lymphocytes prepared as described above.

ISH in buffer (a): labeled DNA probe (pUC probe (1)–(3), DYZ1 probe (1)): approx. 70 ng, each; buffer (a): 3 µl; 20 × standard saline citrate (SSC): 3 µl; H₂O (Milli-Q ZFMQ 23044, Millipore, Eschborn, F.R.G.) to make up a final volume of 30 µl. The hybridization mixture was pipetted on a microscope slide with the fixed metaphase spreads, covered with a cover glass (20 × 40 mm²) and sealed with Fixogum (Marabu, Tamm, F.R.G.). The slide was placed in a specially designed stainless steel chamber, denaturation was done at 94 °C for 5 min in a water bath and the slide was cooled down to 40 °C at room temperature overnight.

ISH in buffer (b): ISH was carried out according to ISH protocol of buffer (a) (see above) with the following modifications: labeled DNA probe: pUC probe (4), total genomic human male DNA; 70 ng and 360 ng, each; buffer (c): 10 µl; 20 × SSC: 5 µl; H₂O to make up a final volume of 50 µl; denaturation at 89 °C for 5 min, then placed in a water bath having an initial temperature of 72 °C which was cooled down to 40 °C at room temperature overnight.

ISH in buffer (c): ISH was carried out according to ISH protocol of buffer (a) (see above) with the following modifications: labeled DNA probe: DYZ1 probe (2); buffer (c): 3 µl; denaturation: 91 °C.

Additionally, total plasmid DNA pUC 1.77 (1.2 µg) (pUC probe (5)) was labeled with DIG-11-dUTP by nick translation performed in buffer (b) (final volume 50 µl), using digoxigenin DNA-labeling mixture (Boehringer Mannheim, Mannheim, F.R.G.) according to product informations and 2 units DNase/polymerase 1 mix (GIBCO BRL, Eggenstein-Leopoldshafen, F.R.G.).

With this labeled DNA probe FISH was per-

formed following a method described elsewhere [4].

Detection

If not explicitly stated otherwise for all DNA probes used, the next day after the start of the hybridization the coverslips were removed. The prepared slides were shortly washed (1 min) in 4 × SSC/0.2% Tween 20 at room temperature. Fluorescence labeling was done with the anti-digoxigenin-fluorescein, Fab fragments (FITC conjugate) (Boehringer Mannheim, Mannheim, F.R.G.). The procedure was performed according to the standard protocol [7] except that after the blocking step the bovine serum albumin (BSA, Serva, Heidelberg, F.R.G.) was removed throughout the remaining washing steps; counterstaining with PI (1.5 µmol/l or 15 µmol/l) and DAPI (5 µmol/l). Additionally after performing ISH in buffer (a) with pUC probe (1) overnight, in one experiment, the slide was incubated in the same stainless steel chamber at room temperature for two weeks before FITC detection (Fig. 2e).

All hybridizations were analyzed with a fluorescence microscope (Orthoplan equipped with a PL APO oil objective 63 ×, numerical aperture 1.40, Leitz, Wetzlar, F.R.G.). Microphotographs (Fujichrom P1600 D) of the hybridized material were mainly taken with a final microscope image magnification of about 630 ×.

*Fluorescence *in situ* hybridization efficiency*

From the beginning of the hybridization region at the left and the right small sites of a slide, metaphase spreads (hybridized and not hybridized) were counted along parallel lines to the large sites of the slide, until a number of 200 metaphase spreads was reached. The efficiency in % was then determined by:

$$\text{efficiency [%]} = \frac{\text{number of metaphase spreads with clear hybrid. sign.}}{200} \times 100.$$

Gel electrophoresis

DNA probe preparations were confirmed by gel electrophoresis using aliquots of 10 µl in a 2% agarose gel (Serva, Heidelberg, F.R.G.), together with two molecular weight markers (DNA molecular weight markers III, V, Boehringer Mannheim, Mannheim, F.R.G.). The electrophoresis buffer consisted of 40 mmol/l Tris-acetate (Carl Roth

GmbH, Karlsruhe, F.R.G.), 1 mmol/l EDTA (Serva, Heidelberg, F.R.G.), pH 8.0. The final concentration of ethidium bromide was in each case 0.5 µg/ml. Electrophoresis was run at a constant field of 3.16 V/cm for 20–45 min. Afterwards the gel was photographed under UV illumination (wavelength: 260 nm).

Results

For repetitive probe preparations (amplification and labeling) a PCR buffer described elsewhere [15] was applied, however a higher Mg^{2+} concentration was used. This allowed an immediate nick translation directly in the amplification product (after PCR), by adding enzyme mix and modified nucleotides (Fig. 1a, lane 4). In the following, this protocol is referred to as "pUC probe (1)". Furthermore, amplification and simultaneous labeling with DIG-11-dUTP by PCR has been performed (Fig. 1a, lane 2); this protocol is referred to as pUC probe (2). These two probes have been used for ISH experiments according to the new hybridization protocol with buffer (a). Additionally a "standard" hybridization protocol using "pUC probe (5)" was performed in order to compare the specific hybridization regions of the DNA probe pUC 1.77 as described [16]. The hybridization signals on two of the largest chromosomes of a metaphase plate (Fig. 2a, arrowheads) indicate the well-known main binding site of this probe in the q 12 region of the human chromosome # 1.

Applying the new hybridization protocol in buffer (a) using "pUC probe (1)" and the "pUC probe (2)", the same main binding sites were obtained (Fig. 2b, c; arrowheads). However, additional hybridization signals were observed (Fig. 2b, c), which are comparable to the minor binding sites already described [16].

Fig. 2d represents a result of FISH according to the new protocol using "pUC probe (3)", (PCR amplification and simultaneous incorporation of DIG-11-dUTP followed by DNase treatment). This resulted in a considerable reduction of the length of the DNA probe (compare Fig. 1, lane 2 with Fig. 1, lane 3). Again hybridization signals were observed comparable to the known major and minor binding sites of the DNA probe pUC 1.77 (compare Fig. 2d with Fig. 2b, c). Chromosomal morphology and ISH signals appeared well preserved or even improved after a two weeks

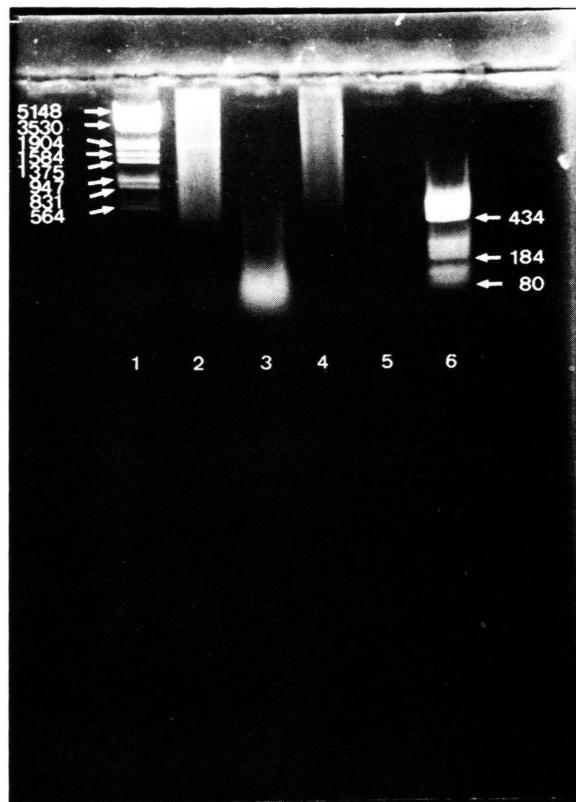
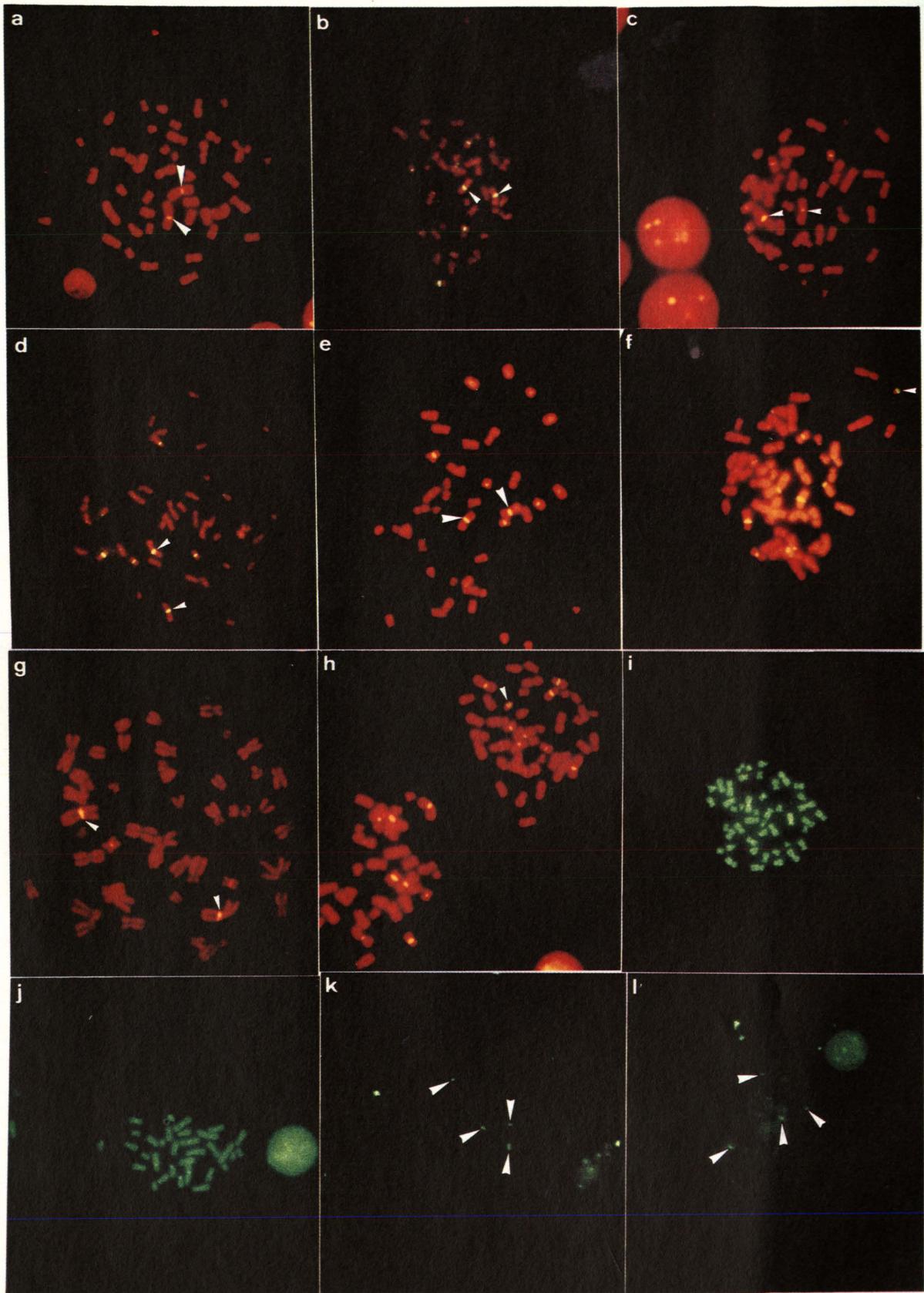


Fig. 1. Photograph of a 2% agarose gel (ethidium bromide, 0.5 µg/ml). Lane 1: marker DNA; the length of the DNA is represented by bands expressed in numbers of basepairs at the left. Lane 2: 10 µl of 50 µl of the solution of the amplified and simultaneously labeled (DIG-11-dUTP) pUC 1.77 DNA by PCR: pUC probe (2). As the amplification template the total pUC 9 plasmid with the human 1.77 kb insert (pUC 1.77) in combination with specific sequencing and reverse sequencing primers was used [14]. A weak band, in the region of 1.77 kb is accompanied by a wide "smear" of DNA fragments caused by different lengths and/or different grade of incorporation of modified nucleotides (DIG-11-dUTP). Due to the incorporation of DIG-11-dUTP, the 1.77 kb fragment has a reduced migration rate [14]. Lane 3: 10 µl of pUC 1.77 DNA, amplified and simultaneously labeled by PCR followed by DNase treatment (pUC probe (3)). Lane 4: 10 µl of pUC 1.77 DNA amplified by PCR and labeled by modified nick translation (pUC probe (1)). In spite of nick translation a wide "smear" indicates long DNA fragments and/or high grade of incorporated modified nucleotides. Lane 5: 10 µl of DYZ1 DNA amplified and simultaneously labeled (DIG-11-dUTP) by PCR, from genomic male human DNA as the template, using specific sequencing primers [17] (DYZ1 probe (1)). Lane 6: marker DNA V (Boehringer Mannheim, Mannheim, F.R.G.): The lengths of the DNA are represented by bands expressed in numbers of basepairs at the right.



period incubation at room temperature (Fig. 2e, compare Fig. 2b).

Additional hybridizations in buffer (a) have been performed with a second repetitive DNA probe. For this purpose the DNA probe DYZ1, specific mainly to the constitutive heterochromatin of the human male Y chromosome [17] was amplified and labeled simultaneously by PCR with DIG-11-dUTP (referred to as "DYZ1 probe (1)"). Amplification was confirmed by gel electrophoresis (Fig. 1, lane 5).

All hybridized human male metaphase plates showed on one of their smallest chromosomes hybridization signals (yellowish-green spots) as expected for ISH of the constitutive heterochromatin of the human male Y chromosome (Fig. 2f, arrowhead). Furthermore additional hybridization signals on a number of different chromosomes have

been observed, apparently located mainly around the centromeric regions (Fig. 2f).

For all hybridizations according to the new protocol in buffer (a) using both DNA probes (Fig. 2a–f), an apparently low fluorescence background was observed. For these cases a mean hybridization efficiency of 99.4% (97.5%–100% at 5 randomly selected slides) was estimated in the hybridization areas.

In a next step we investigated if such a close combination of DNA probe preparation and ISH is restricted to this special PCR buffer systems (buffer (a)) only.

For probe preparation and ISH two further buffers (buffer (b), (c)) were used together with the two repetitive DNA probes pUC 1.77 and DYZ1, hybridized to chromosomes fixed on slides obtained from human lymphocytes.

Fig. 2. Microphotographs of metaphase spreads obtained from human lymphocytes, hybridized in different buffer systems according to the technique described in this report, except experiments represented by Fig. 2a. The different DNA probes were labeled with DIG-11-dUTP. Detection of ISH by indirect immunofluorescence with anti-DIG-fluorescein conjugate. Counterstaining, if not stated otherwise, with propidium iodide (15 $\mu\text{mol/l}$) and DAPI (5 $\mu\text{mol/l}$).

- a: "Standard" ISH using formamide (60%), with pUC probe (5). Two hybridization signals in the centromeric regions on two of the largest chromosomes are visible (yellowish-green colour, arrowheads), representing the major binding site of the probe, located in the q12 region of human chromosome # 1.
- b: ISH in buffer (a), using pUC probe (1) (PCR amplified and labeled by modified nick translation). The major binding site is indicated by yellowish-green signals (arrowheads). Additionally, minor binding sites are visible. Counterstaining with propidium iodide (1.5 $\mu\text{mol/l}$).
- c: ISH in buffer (a), using pUC probe (2) (PCR amplified and simultaneously labeled in buffer (a)). Major binding (arrowheads) and minor binding sites are visible (compare 2b).
- d: ISH in buffer (a), using pUC probe (3) (probe DNA preparation, see 2c, with additional DNase treatment afterwards). Major binding (arrowheads) and minor binding sites are visible (compare 2b and 2c).
- e: ISH in buffer (a), probe preparation see 2c; however, the slide with the hybridized metaphase spreads was stored for two weeks in the hybridization mixture at room temperature, followed by indirect immunostaining. Major binding (arrowheads) and minor binding sites are visible (compare 2b and 2c).
- f: ISH in buffer (a), using DYZ1 probe (1) (PCR amplified and simultaneously labeled in buffer (a)). Major binding site, human Y chromosome, indicated by a single yellowish-green signal (arrowhead). Additional minor binding sites are visible. Counterstaining with propidium iodide (1.5 $\mu\text{mol/l}$).
- g: ISH in buffer (b), using pUC probe (4) (PCR amplified and labeled by modified nick translation). The locations of the major binding sites are indicated by arrowheads; only one minor binding site is visible (compare 2b and 2c).
- h: ISH in buffer (c), using DYZ1 probe (2) (PCR amplified and simultaneously labeled in buffer (c)). The major binding site is indicated by a single yellowish-green signal (arrowhead) (compare 2f).
- i: ISH in buffer (b), using human genomic male DNA (labeled by standard nick translation in a standard buffer system). All chromosomes of the metaphase plate show a nearly homogenous FITC staining.
- j: ISH in buffer (b), using only $\frac{1}{5}$ of the human genomic male DNA applied in 2i. Chromosomes show weaker staining compared to 2i, except in some cases the centromeric regions are stained more intensively.
- k: ISH in buffer (b). The ISH procedure was performed under the same conditions as in the experiments represented in 2i (PCR amplified and simultaneously labeled in buffer (b), using pUC probe (2)). No counterstaining with propidium iodide was performed; therefore the metaphase spread appears only as a shadow; however, four hybridization signals (arrowheads) are visible.
- l: ISH in buffer (b), using only $\frac{1}{5}$ of the pUC probe (2) in 2k. The ISH procedure was performed under the same conditions as in the experiment represented in 2j. Four hybridization signals are visible, compare 2k, indicating specific hybridizations of this probe. Because the same conditions (except probe preparation) were used in 2i/2k and 2j/2l, respectively, the general FITC staining of all chromosomes in 2i and 2j is not caused by non-incorporated labeled nucleotides.

High amplifications of either pUC 1.77 in buffer (b) or DYZ1 in buffer (c) were obtained; these combinations were used for FISH, too. In both cases comparable results to FISH using buffer (a) were observed (Fig. 2g, arrowheads; 2h, arrowhead). In the case of pUC probe (4) (buffer (b)) however, additionally only one minor binding site of the DNA probe pUC 1.77 was detected, suggesting a higher stringency of this buffer. Amplification of the DYZ1 DNA probe in buffer (b) did not yield to good results.

Furthermore, it was examined whether this technique of using PCR buffers for ISH is restricted to repetitive probes only. Therefore we used total human male genomic DNA labeled by standard nick translation with DIG-11-dUTP for hybridization to male metaphase spreads according to the technique described above in buffer (b). Fig. 2i represents metaphase spread after FISH without counterstaining. A nearly completely "FITC staining" was observed. Since the hapten modified nucleotides in the buffer had not been removed prior to ISH, the complete FITC staining might have been caused by these modified nucleotides. For this reason other experiments were performed. A second FISH was carried out applying only $\frac{1}{5}$ of the previously used labeled male human DNA (Fig. 2j). This resulted in a reduction of chromosome staining (labeling) whereas higher FITC fluorescence intensity has been observed mainly in centromeric regions of many of the metaphase chromosomes.

Additional *in situ* hybridizations under the same conditions have been carried out using DNA probe pUC 1.77 ("pUC probe (2)"). Only four FITC signals on each metaphase spread were detected (Fig. 2k, 2l; arrowheads) whereas FITC fluorescence was hardly observed at the remaining chromosome regions (chromosomes). This indicates a very low "hybridization efficiency" of the non-incorporated modified nucleotides during ISH.

Discussion

In this report we have shown that in PCR buffer systems a close combination of DNA amplification, labeling and ISH on chromosomes of human lymphocyte metaphase spreads is possible. This was shown with two repetitive DNA probes (pUC 1.77, DYZ1) using three different PCR buffers.

In this technique, the buffers used for PCR amplification and labeling may also be used in an almost identical composition for *in situ* hybridizations. The only change made for, was the addition of SSC (standard saline citrate) to the PCR buffer. Moreover, the latest experiments with this technique, showed that hybridization is accomplished within 30 min, thus making overnight incubation unnecessary (manuscript in preparation).

The results indicate that these ISH techniques conserves the specificity of the repetitive DNA probes used here. This was shown for pUC 1.77 (main binding site 1q12) by comparison of the ISH obtained with this method, with the ISH by a "standard" technique using formamide (see experiments DNA probe 1.77: pUC probe (1)–(4) and pUC probe (5)). In both cases FITC fluorescence at the centromeric regions on two of the largest chromosomes of a metaphase plate was observed. Using the new procedure, however, additional hybridization sites (FITC fluorescence) were found mainly in centromeric regions of other chromosomes, comparable to the minor binding sites known for this probe [16]. This may perhaps indicate a lower stringency using PCR buffers. However, in all experiments the hybridization signals of the major binding site of the pUC 1.77 probe were considerably stronger than the signals of the minor binding sites (as ascertained from direct observations). The combination of ISH with different probe preparations (modified nick translation, standard nick translation, simultaneous labeling by PCR), appears to have small effects on the results (see Fig. 2). Even an incubation period of two weeks at room temperature prior to FITC detection showed no visible changes (see Fig. 2e).

Comparable results were obtained using DYZ1 as a second DNA probe (specific mainly for the heterochromatin at the Y chromosome). On one of the smallest chromosomes of a human male metaphase spread, FITC fluorescence was detected indicating the known major binding site of the DZY1 probe on the Y chromosome [17]. Also in this case, however, a "lower stringency" using PCR buffers was indicated by an additional number of minor binding sites in centromeric regions of other chromosomes. Such binding sites were also observed by other authors [17] (U. Weier, personal communication 1991). All FISH experiments using these two repetitive DNA probes

showed a comparable hybridization efficiency of about 99.4% (97.5%–100%) in the hybridization areas, accompanied by a low FITC background and a well preserved chromosomal morphology.

Furthermore, FISH experiments using whole genomic DNA as the probe suggest that this technique may not be restricted to the repetitive DNA probes used here. Further investigations concerning this matter, however, are required.

In summary we here present a method which combines probe amplification, labeling and ISH in PCR buffer systems. Moreover these techniques may simplify also FISH in suspension. Due to the lack of formamide or other denaturing agents, necessary washing steps may be minimized. This results in a reduction of centrifugal steps, leading to a considerably higher amount of hybridized chromosomal material. Such an improvement of FISH in suspension is interesting for a variety of applications, such as magnetic sorting [18] and slit-scan flow cytometry [19].

In contrast to the FISH technique described in this report, conventional FISH protocols require relatively large quantities of formamide (in the order of 0.21 per preparation). Although formamide is regarded a weakly toxic substance, its use may become an environmental hazard in routine application. We are now investigating techniques which can be performed under nearly physiological conditions (temperature, buffer composition), based on triple helix formation.

Acknowledgements

We thank Prof. Dr. T. Cremer, Institute of Human Genetics and Anthropology, University of Heidelberg, for providing the pUC 1.77 DNA probe and the human lymphocyte preparations. The work was supported by the Deutsche Forschungsgemeinschaft.

- [1] T. Cremer, P. Lichter, J. Borden, D. C. Ward, and L. Manuelidis, *Hum. Genet.* **80**, 235–246 (1988).
- [2] P. Lichter, T. Cremer, C. J. Chang Tang, P. C. Watkins, L. Manuelidis, and D. C. Ward, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9664–9668 (1988).
- [3] D. Pinkel, J. Landegent, C. Collins, J. Fuscoe, R. Segraves, J. Lucas, and J. W. Gray, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9138–9142 (1988).
- [4] P. Emmerich, P. Loos, A. Jauch, A. H. N. Hopman, J. Wiegandt, M. J. Higgins, B. N. White, M. van der Ploeg, C. Cremer, and T. Cremer, *Exp. Cell Res.* **181**, 126–149 (1989).
- [5] J. Anastasi, M. M. Le Beau, J. W. Vardiman, and C. A. Westbrook, *Am. J. Pathol.* **136**, 131–139 (1990).
- [6] T. Cremer, S. Popp, P. Lichter, and C. Cremer, *Cytometry* **11**, 110–118 (1990).
- [7] P. Lichter, C. C. Tang, K. Call, G. Hermanson, H. J. Evans, D. Housman, and D. C. Ward, *Science* **247**, 64–69 (1990).
- [8] J. Wienberg, A. Jauch, R. Stanyon, and T. Cremer, *Genomics* **8**, 347–350 (1990).
- [9] P. Lichter, A. L. Boyle, T. Cremer, and D. C. Ward, *Genet. Anal. Techn. Appl.* **8**, 24–35 (1991).
- [10] C. Cremer and T. Cremer, *Europ. J. of Histochem.* **36**, 15–25 (1992).
- [11] G. Dudin, T. Cremer, M. Schardin, F. Bier, and C. Cremer, *Hum. Genet.* **76**, 290–292 (1987).
- [12] C. Lengauer, H. Riethman, and T. Cremer, *Hum. Genet.* **86**, 1–6 (1992).
- [13] I. Dunham, C. Lengauer, T. Cremer, and T. Featherstone, *Hum. Genet.*, in press.
- [14] D. Celeda, U. Bettag, and C. Cremer, *Biotechniques* **12**, 98–102 (1992).
- [15] J. Sambrook, E. F. Fritsch, and T. Maniatis, *Cold Spring Harbor Laboratory Press, Molecular Cloning* (1989).
- [16] J. R. Gosden, S. S. Lawrie, and H. J. Cooke, *Cell Genet.* **29**, 32–39 (1981).
- [17] H. U. G. Weier, R. Segraves, D. Pinkel, and W. J. Gray, *J. Histochem. Cytochem.* **38**, 421–426 (1990).
- [18] G. Dudin, E. W. Steegmayer, P. Vogt, H. Schnitzer, E. Diaz, K. E. Howell, T. Cremer, and C. Cremer, *Hum. Genet.* **80**, 111–116 (1988).
- [19] M. Hausmann, G. Dudin, J. A. Aten, R. Heilig, E. Diaz, and C. Cremer, *Z. Naturforsch.* **46**, 433–441 (1991).
- [20] A. Boyum, *Scand. J. Clin. Invest.* **21**, 97 (1968).