Human Cytogenetics

Volume I
Constitutional Analysis

A Practical Approach

Edited by

D. E. ROONEY

Cytogenetics Unit,
St Mary's Hospital Medical School,
Norfolk Place, London W2 1PG, UK

and

B. H. CZEPULKOWSKI

Department of Haematology,
King's College Hospital,
Denmark Hill, London SE5 8RX, UK

IRL PRESS
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OXFORD UNIVERSITY PRESS
Oxford New York Tokyo

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Chromosome analysis by non-isotopic *in situ* hybridization

P. LICHTER and T. CREMER

1. Introduction

In situ hybridization provides the most direct way to study the chromosomal localization of DNA sequences. Based on the early work of Gall and Pardue in 1969 (1), routine isotopic *in situ* hybridization protocols were established in many laboratories during the 1970s. In 1981 Gerhard *et al.*, Malcolm *et al.*, and Harper *et al.* were the first to demonstrate the possibility of localizing single-copy sequences cloned from individual genes by isotopic *in situ* hybridization (2–4). A protocol for isotopic chromosomal localization of DNA probes is described elsewhere in this *Practical Approach* series (5). This chapter is devoted to non-isotopic *in situ* hybridization techniques, in particular, fluorescence *in situ* hybridization protocols. Based on the developments in the early 1980s (for review, see ref. 6), this approach has become increasingly popular over the last few years. A number of reasons account for this development (for discussion, see refs 7–8), notably an increase in speed, improved signal resolution, the development of *in situ* suppression hybridization protocols, advanced optical equipment for 2-D and 3-D analyses of labelled specimens, and in particular the ability to combine several techniques for simultaneous multicolour detection of different nucleic acid targets within the same preparation. *Figure 1* illustrates schematically the principles of this method.

Non-isotopic *in situ* hybridization using chromosome specific DNA probes provides a powerful tool for the analysis of numerical and structural chromosome aberrations not only in metaphase, but also directly in the interphase nucleus, an approach which has been termed *interphase cytogenetics* (9) (for review, see ref. 8). This is of great significance in cases where metaphase chromosome spreads cannot be prepared in sufficient quality or quantity (e.g. as is the case with many tumours), or not at all as in non-cycling cells.

The first applications of non-isotopic *in situ* hybridization to clinical cytogenetics took advantage of tandemly repeated, chromosome specific DNA elements, such as alphoid DNA sequences, which are present in the heterochromatin of most human chromosomes (for review, see ref. 8). A procedure

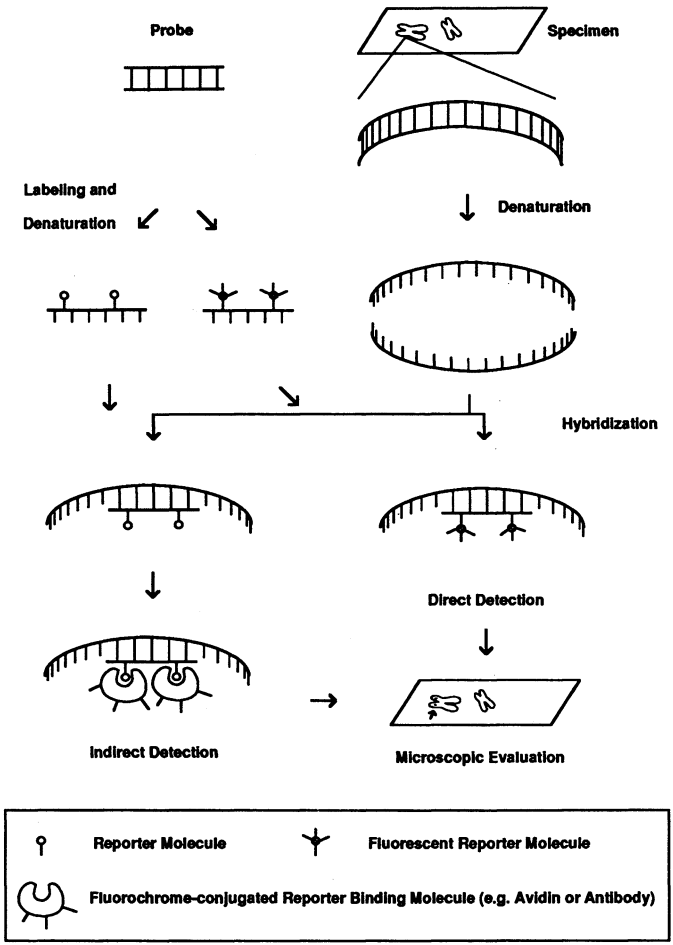


Figure 1. Schematic illustration of fluorescence *in situ* hybridization.

useful for such purposes is given below (*Protocols 3–6*). Since the target region is considerably large, expanding up to many hundred kilobases in length, it is easy to detect after *in situ* hybridization with a single repeated element or even part of it used as a probe. Recently, oligomeres defining highly specific sub-sequences of the repeated element have been introduced as probes. The latter approach, however, requires special protocols of oligomere synthesis or enzymatic tailing in the presence of chemically modified nucleotides, which are beyond the scope of this chapter.

Typical probes for mapping to human chromosomes are either cDNAs or cloned genomic DNA fragments. Most cDNAs consist only of single-copy sequences whereas the vast majority of cloned genomic fragments contain, in addition to unique sequences, interspersed repetitive sequences (IRS), such as SINEs (small interspersed repetitive elements, e.g. Alu-elements) and LINEs (large interspersed repetitive elements, e.g. L1-elements). For cDNAs and genomic fragments known to be entirely single-copy the standard procedure (*Protocols 3–6*) can be used. Parameters influencing the quality of non-isotopic *in situ* hybridization signals have been optimized and more precisely defined by a number of groups (e.g. refs 10–15). All this knowledge has contributed to the standard procedure described.

When interspersed repetitive elements are present in a probe, application of the standard procedure results in hybridization signals distributed over the whole chromosome complement due to the ubiquitous presence of IRS throughout the genome. Since the most abundant repetitive DNA is the Alu-repeat family, which is preferentially located within R-bands (16), usually an R-banding-like staining pattern results. Protocols have been developed by which the portion of the signal caused by IRS within a given probe can be suppressed, while a highly specific signal is produced by the target site specific single-copy (or low-copy repeat) sequences (17–19). The principle of this method, which has been called *chromosomal* in situ *suppression* (CISS) *hybridization* (18), is illustrated in *Figure 2*. The labelled probe fragments are denatured together with an excess of unlabelled competitor DNA. Thereafter, pre-annealing is allowed for a given period of time. During this time, IRS elements of the probe will hybridize rapidly with the excess IRS of the competitor DNA, while most of the much less abundant chromosome specific sequences remain single-stranded. Accordingly, the resulting double-stranded IRS elements in the pre-annealed probe can no longer hybridize to their chromosomal DNA targets. In addition, a second competition effect is likely to contribute to the suppression of IRS signals. Remaining single-stranded IRS elements will hybridize to chromosomal IRS targets during the subsequent *in situ* hybridization. Again, unlabelled IRS elements of the competitor DNA would be in excess, and hybridization of remaining, single-stranded probe IRS elements would be prevented (see bottom of *Figure 2*). Any source of human DNA containing a sufficiently high concentration of IRS may serve as competitor DNA. Routinely, we use total genomic DNA (e.g. placental

DNA). In this case, too much competitor DNA is disadvantageous since chromosome site specific signals might also become significantly suppressed in addition to IRS signals. This risk can be avoided by using the *Cot1* fraction of human genomic DNA, since the latter consists largely of rapidly annealing repetitive elements. *Cot1* fractions have recently become commercially available. Salmon sperm DNA is added to the pre-annealing reaction in order to adjust the total DNA concentration. When there is still considerable staining of the chromosomes after *in situ* hybridization caused by IRS sequences, the experiment should be repeated with more competitor. However, some IRS banding can be helpful for chromosome identification. A protocol for CISS hybridization is given below (*Protocol 8*).

There is a fairly good correlation of labelling efficiency and size of targeted DNA. Cosmids commonly contain 25–40 kb of cloned insert DNA and are very efficient probes which almost all yield highly specific signals on metaphase chromosomes. Usually more than 90% of all metaphases show signals on both chromatids of both chromosome homologues (see *Figure 3*) with virtually no background spots. Therefore, the need for statistical analysis is greatly reduced and the efficiency is high enough to use such probes for diagnosis of chromosomal aberrations such as trisomies (see *Figure 3*) (20), translocations (21–23), inversions (24), and deletions (23, 25, 26). When the size of the target DNA detected by a given probe decreases (e.g. using probe fragments cloned in phage or plasmid vectors), the percentage of successfully delineated target sites also decreases. With probes containing approximately 2 kb single-copy sequences, a maximum of 40 to 50% of all target sites can presently be detected. Although we and others have successfully mapped single-copy DNA fragments of about 1 kb by fluorescence *in situ* hybridization, the discrimination of weak specific signals against background dots is likely to require statistical analysis based on the time-consuming evaluation of many metaphases. The development of very powerful camera systems which are more sensitive than the human eye promises to improve the detection of very weak hybridization signals significantly (see Section 6). Alternatively, it may be advisable to first isolate a larger probe for physical mapping, for instance, by screening a cosmid library.

The mapping of yeast artificial chromosomes (YACs) can also be carried out applying CISS hybridization (23, 27). Total genomic DNA of yeast cultures containing YACs with human DNA fragments has been successfully used as a probe. In this case, the amount of probe DNA has to be increased due to the small percentage of YAC DNA in yeast cells, while the concentration of human competitor DNA remains constant. Total yeast DNA can be added as competitor but is not essential. The quality of *in situ* hybridizations with YAC probes is often increased when YAC DNA purified after pulsed field gel electrophoresis is used. An alternative technique for generating YAC DNA probe sequences is described below (see Section 7).

In order to stain whole chromosomes or larger chromosomal regions,

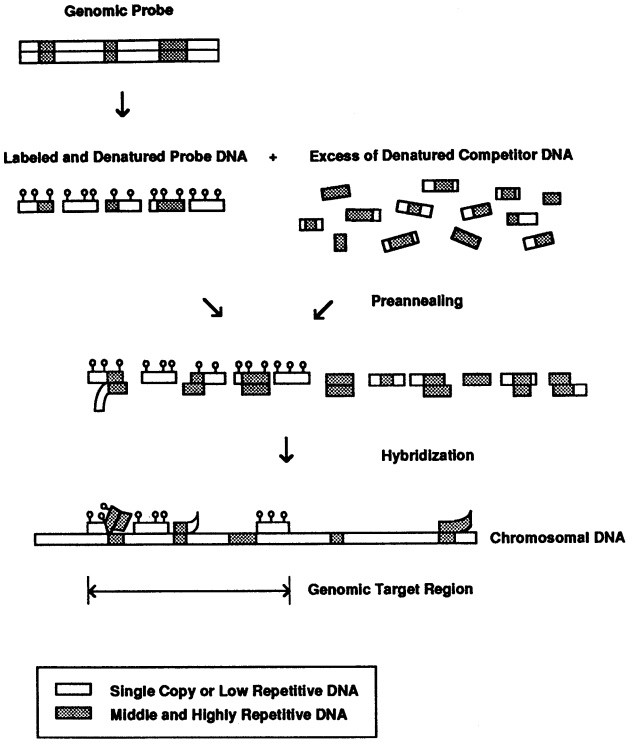
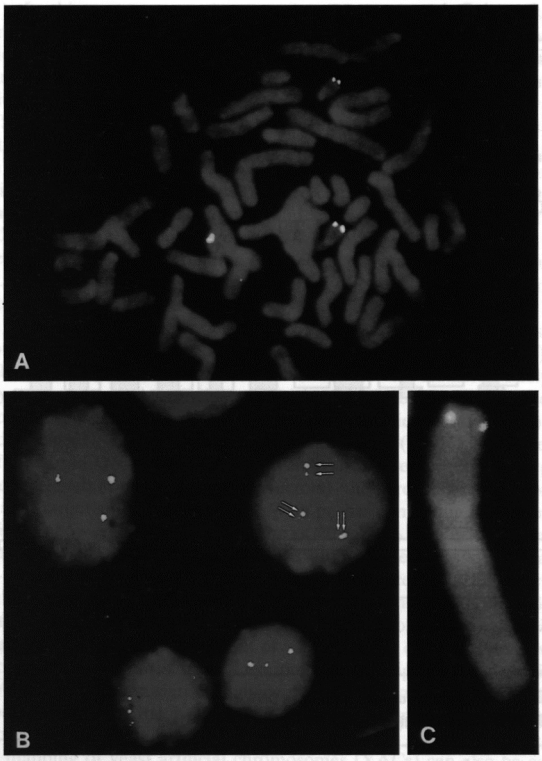


Figure 2. Schematic illustration of chromosomal *in situ* suppression (CISS) hybridization. For explanation see text.

different kinds of probe sets can be used. Entire chromosomes are specifically delineated using the DNA from sorted human chromosomes as a pool (18, 19). This procedure has also been termed 'chromosome painting' (19). The libraries used are either cloned in lambda phage vectors (28) or in pBS plasmid vectors (29). The latter are generally easier to use since the portion of vector sequences is small compared to most of the presently available recombinant lambda libraries. Accordingly, background problems which may arise



from high amounts of labelled vector can be eliminated. This problem could otherwise be overcome by isolating the library inserts (18). *Figure 6*A (p. 186) shows a chromosome 5 of *Macaca fuscata* specifically painted with total bacteriophage library DNA derived from human chromosome 5. Painting of the complete homologous *Macaca* chromosome 5 indicates the high level of evolutionary conservation of these chromosomes in human and *Macaca fuscata* for at least twenty million years.

Probe sets for the specific staining of subchromosomal regions can be generated in several ways. These include the pooling of single clones from

Figure 3. Chromosomal *in situ* suppression hybridization with cosmid DNA probes. (A and B) Cosmid c512 mapping to 21q22.3 hybridized to a metaphase spread (A) and nuclei (B) of lymphocytes from a Down's syndrome patient. The biotin labelled probe was detected with avidin–FITC; chromosomes and nuclei were counterstained with propidium iodide. Note the highly specific labelling, allowing a rapid diagnosis of trisomy 21 (20). On the metaphase in panel A the signals are visible on both chromatids (all signals are doublets) of all three chromosomes 21. Correspondingly three signals are seen in the nuclei (B). Note that the signals in the nuclei are doublets also (depicted, for example, by the small arrows in the upper right nucleus), indicating the G_2 stage of these nuclei. The doublets are not always visible simultaneously in G_2 nuclei, since part of the fluorescence spots are not in the imaged focal plane. (C) Cosmid K40 containing the human haemoglobin beta gene cluster localized on the short arm of chromosome 11 (42). Note the pale background R-banding pattern by propidium iodide in A and C. (A–C) These digitized images were obtained by using a confocal laser scanning microscope and after applying image processing for better illustration (see Section 6).

a particular region (30), regional specific DNA libraries established from microdissected chromosomal material (31, 32) (*Figure 6C*), as well as genomic DNA of somatic cell hybrids containing pieces of human chromosomes (e.g. 'radiation induced hybrids') (33, 34). Alternatively, species-specific polymerase chain reaction (PCR) amplification products from such hybrids constitute probe sets of high quality (see also Section 7).

2. Specimen preparation

A number of specimen preparation techniques are compatible with fluorescence *in situ* hybridization. For specimen preparation for more specialized applications (such as studies of numerical and structural chromosome aberrations in tissue sections from solid tumours and three-dimensional chromosome analysis directly in the cell nucleus) the reader is referred to the literature (35–37). Painting procedures and mapping of DNA probes on metaphase chromosomes can be carried out using conventional methanol/acetic acid fixed metaphase spreads (see Chapter 2, Section 2). After chromosome spreading, the air-dried preparations are dehydrated in the following way:

(a) Put slides through a series of 70%, 90%, and 100% ethanol (10 min each) at room temperature.

(b) Air-dry again.

(c) Put slides in containers and keep at room temperature for short-term use.

or

Seal the containers in bags containing drierite and keep at −70°C (or −20°C) for long-term storage. In our experience slides can be stored at −70°C for at least one year without affecting their quality for *in situ* hybridization. Once a container has been thawed do not refreeze the slides.

Slides can also be stored in 70% ethanol for up to several weeks. Although it is generally believed that slides should age several days before performing an *in situ* hybridization, we have achieved good results with slides prepared the same day. However, in these cases baking of the slides for 2–3 h at 50–60°C has been proved to better conserve chromosome morphology.

3. Labelling procedures

A number of sensitive non-isotopic labelling and detection procedures are available (for review see refs 6, 8). Because of their high sensitivity and commercial availability two techniques are routinely used in many laboratories: probe labelling with either biotin or digoxigenin as reporter molecules and indirect detection of hybridized probe via avidin or antibodies, (see right half of *Figure 1*). As nucleotides conjugated directly with fluorescent dyes are becoming commercially available, the *in situ* hybridization and direct detection of fluorochromated probes (see left half of *Figure 1*) provides an alternative procedure, which is particularly useful when large regions are targeted, i.e. for staining of repetitive DNA or for chromosome painting. Recently, several suppliers have started to offer chromosome specific probes labelled with biotin or fluorochromes. The commercial availability of probes for chromosomes and chromosomal sub-regions of interest in combination with commercial *in situ* hybridization and detection kits will facilitate the introduction of the new staining procedures even in cytogenetic laboratories which are not generally equipped for molecular cytogenetics.

Although a vast majority of vector sequences in a pool of probes can cause an increase in background, in the case of single probes there is usually no need for insert isolation. In our experience, the isolation of inserts from plasmid vectors, for example, prior to the labelling does not improve the results. It has been postulated that some vector sequences within a probe will facilitate the formation of a network of probe fragments at the site of hybridization and thus increase the signal intensity.

Labelling by enzymatic incorporation of modified nucleotides is much more efficient than labelling by incubation with reactive compounds, such as photobiotin. Prokaryotic polymerases used for different nucleic acid labelling procedures accept unmodified nucleotides better than they accept modified nucleotides. Therefore they incorporate modified nucleotides only up to a certain level. In our experience, the non-isotopic labelling efficiency of primer extension (38) and nick-translation (see *Protocol 1*) is comparable. Another important requirement for a good *in situ* hybridization probe is the size of the probe molecules obtained after the labelling reaction. The molecules should be between 100 and 500 nucleotides in length. This size range can be easily obtained by adjusting the DNase concentration in a nick-translation reaction. Therefore, we prefer nick-translation for labelling, especially because long

genomic fragments as template in a primer extension reaction often result in probe molecules of a higher size-range.

3.1 Biotinylation by nick-translation

This protocol is based on the work of Langer *et al.* (39). Since labelled probes can be stored for long periods (at −20°C) without affecting the probe quality, large probe amounts can be labelled at one time. A standard reaction is carried out with 2 μg template DNA in 100 μl volume. The solution with the prepared DNA template should be free of RNA. During the establishment of the labelling procedure in a laboratory, when new reagents are prepared, or when no *in situ* hybridization signal can be found, it is advisable to perform a dot-blot assay to test the biotin labelling (*Protocol 2*).

Protocol 1. Nick-translation

Materials

- 10 × buffer containing 0.5 M Tris–HCl pH 8.0, 50 mM $MgCl_2$, 0.5 mg/ml BSA.
- 0.1 M β-mercaptoethanol. Dilute 0.1 ml of β-mercaptoethanol with 14.4 ml double-distilled water.
- 10 × nucleotide stock containing 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP and 0.5 mM biotin-11-dUTP.[a]
- DNase I solution. Dissolve 3 mg DNase I in 0.5 ml 0.3 M NaCl, add 0.5 ml glycerol, store at −20°C. Dilute 1 μl of this stock solution in 1 ml of ice-cold water *immediately* before use.[b]
- Column buffer containing 10 mM Tris–HCl pH 8.0, 1 mM EDTA and 0.1% SDS.
- Resin for spin column. Disperse 30 g of Sephadex G-50 (medium) in 300 ml of column buffer containing 0.1% SDS[c] and incubate for several hours at 95°C or autoclave.

Method

1. Combine:
   - RNA-free DNA solution containing 2 μg DNA
   - 10 μl of 10 × buffer
   - 10 μl of 0.1 M β-mercaptoethanol
   - 10 μl 10 × nucleotide stock
   - tested volume of DNase I solution[b]
   - 20 units *Escherichia coli* DNA polymerase I

   adjust to 100 μl with double-distilled water (enzymes should be added last)

Protocol 1. *Continued*

2. Incubate the reaction for 2 h in a 15°C water bath.
3. Put the reaction on ice, and keep it on ice until the actual size of the probe molecules is determined.
4. Take an aliquot of 7 μl, add gel loading buffer to the aliquot, denature it by incubation in a boiling water bath for 3 min, put the tube on ice for 3 min, load the aliquot on a standard 2% agarose minigel along with a suitable size marker, quickly run the gel (e.g. 15 volts per centimetre for 30 min) to avoid renaturation of the probe in the gel, visualize DNA in the gel, e.g. by staining in 0.5 μg/ml ethidium bromide, and take photographs during UV illumination.
5. The probe molecules will be visible as a smear. This should contain only fragments smaller than 500 nt and larger than 100 nt. A peak intensity at 250–300 nt seems optimal.
   (a) If the DNA is between 100–500 nt, proceed to step 6;
   (b) if the probe is larger, add more DNase I to the reaction kept on ice, incubate further at 15°C (usually higher concentrations of DNase are added for a further 30 min incubation) and repeat the previous step 4;
   (c) if the DNA is not or almost not digested, purify probe and repeat again starting from step 1;
   (d) if part of the DNA is smaller than 100 nt, repeat starting from step 1 using less volume of DNase I dilution;
   (e) if all the DNA is smaller than 100 nt, purify probe from possible contaminating DNase and repeat again starting from step 1.
6. For enzyme inactivation add 3 μl of 0.5 M EDTA (15 mM final concentration), 1 μl 10% SDS (0.1% final concentration), and heat for 15 min at 68°C.
7. Separate labelled probe from unincorporated nucleotides by gel filtration using a spin column.
   (a) Take a 1-ml syringe; remove the stamp; put a plug of silanized glass wool (2–3 mm high) at the bottom of the syringe.
   (b) Pack the syringe with buffered Sephadex G-50 resin to the 1-ml mark.
   (c) Put the column in a 15-ml tube and spin in a clinical centrifuge at 1600 *g* for 5 min.
   (d) Remove flow through, pack again to the 1-ml mark when necessary, spin as before,
   (e) Load 100 μl column buffer and spin again.
   (f) Repeat this step two more times.
   (g) The flow through after the third wash of the column should have the same volume as the loaded buffer, i.e. 100 μl. Put a small reaction tube in the 15-ml tube underneath the syringe, load the sample, and spin as before. The flow through now contains the labelled probe at a

concentration of approximately 20 ng/μl. In this form the labelled probe is ready to use or can be frozen for long-term storage.

Biotinylated DNA should not be subjected to phenol extraction, since the DNA will be lost in the phenol phase or the phenol/water interphase due to the hydrophobicity of biotin.

[a] There are also biotinylated nucleotides other than biotin-11-dUTP commercially available. Linker arms longer than 11 (e.g. biotin-14-dXTP or biotin-16-dXTP) are also appropriate to label *in situ* hybridization probes. When the modified nucleotide is different, e.g. biotinylated dATP, substitute the 0.5 mM dATP with 0.5 mM biotinylated dATP, and 0.5 mM biotin-11-dUTP with 0.5 mM dTTP, respectively.

[b] The volume of the diluted stock that is used in the nick-translation reaction must be tested for each new batch of DNase I stock solution. Carry out a series of digestions with 2 μg of a probe DNA, 10 μl 10 × buffer, 10 μl β-mercaptoethanol, and 1 μl, 2.5 μl, 5 μl or 10 μl of the DNase I dilution, respectively, in a volume of 100 μl. Incubate for 2 h at 15°C. Thereafter, take an aliquot of 7 μl and test the size of the digested DNA as described (step 4 in *Protocol 1*). Choose the volume of DNase that resulted in probe fragments of 100–500 nt in length for the nick-translation reaction (see step 1 in *Protocol 1*). The DNase stock should be diluted (1:1000) immediately before applying to the nick translation reaction. Discard the remainder of the dilution.

[c] SDS has to be included because the biotinylated probe might otherwise stick in the column due to the hydrophobic biotin groups.

Protocol 2. Dot-blot assay to test biotin labelling

This simple colorimetric assay is designed to test the quality of biotinylation of a probe. Commercial kits for this dot-blot assay are available.

Materials

- DNA dilution buffer containing 0.1 mg/ml sheared salmon sperm DNA and 6 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M Na_3 citrate, pH 7.0).
- Prepare a series of dilutions of biotinylated standard DNA (purchased standard or previously used, well-labelled probe) in DNA dilution buffer (see above) resulting in solutions of 0, 1, 2, 5, 10, and 20 pg/μl standard DNA.
- AP 7.5 buffer containing 0.1 M Tris–HCl pH 7.5, 0.1 M NaCl and 2 mM $MgCl_2$.
- AP 9.5 buffer containing 0.1 M Tris–HCl pH 9.5, 0.1 M NaCl and 50 mM $MgCl_2$.
- Blocking buffer containing 3% BSA (fraction V) in AP 7.5 buffer.
- TE buffer containing 10 mM Tris–HCl pH 7.5 and 1 mM EDTA.
- Prepare or buy 75 mg/ml nitroblue tetrazolium in 70% dimethylformamide and 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide.

Method

1. Prepare a series of dilutions of biotinylated test DNA in DNA dilution

Protocol 2. *Continued*

buffer (see above) resulting in solutions of 0, 1, 2, 5, 10, and 20 pg/μl test DNA.

2. Spot 1-μl aliquots of the different concentrations of test DNA in parallel with 1-μl aliquots of the same concentrations of the standard DNA on a small piece of nitrocellulose filter (e.g. 3 × 4 cm).
3. Bake the filter for 30–60 min at 80°C.
4. Wash the filter with AP 7.5 buffer for 1 min at room temperature in a dish.
5. Seal the filter in a plastic bag together with 5–10 ml blocking buffer, and incubate for 30–60 min at 37°C.
6. Open the bag at one end, squeeze out the blocking buffer, fill in a freshly prepared solution of streptavidin-conjugated alkaline phosphatase (dilute streptavidin-conjugated alkaline phosphatase to 1 μg/ml in AP 7.5 buffer), seal the bag again and incubate for 30 min at 37°C.
7. Take the filter out of the bag and wash at room temperature in a dish with AP 7.5 buffer 2 × for 5 min followed by a wash with AP 9.5 buffer for 10 min (agitate).
8. Seal the filter in a plastic bag together with 7.5 ml substrate buffer *freshly* prepared in the following way: Add 33 μl of nitroblue tetrazolium (NBT) to 7.5 ml AP 9.5 buffer, mix gently, add 25 μl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and mix gently again (do not vortex!). Incubate in the dark at 37°C or, alternatively, at room temperature (to slow down the reaction) until colour development is suitable. Usually 15–60 min is sufficient whereas longer incubation often results in higher background.
9. To stop the reaction, take the filter out of the bag and wash at room temperature (agitate) in a dish with TE buffer followed by air-drying.
10. Evaluate the colour signals. In order to be suitable for *in situ* hybridization, the spotted test probe should give signals of similar intensities as the spotted standard DNA of the corresponding concentrations. If the labelling was good and the assay worked well even 1 pg of labelled probe is visible.

3.2 Digoxigenin-labelling by nick-translation

For simultaneous hybridization/dual colour detection usually biotin labelled probes are combined with alternatively labelled probes. A very popular alternative non-isotopic technique is the digoxigenin based detection system (40). Digoxigenin labelling of DNA probes is carried out exactly the same

way as biotinylation by nick-translation (see *Protocol 1*) with one exception: 10 × nucleotide stock contains:

- 0.5 mM dATP
- 0.5 mM dGTP
- 0.5 mM dCTP
- 0.375 mM dTTP, and
- 0.125 mM digoxigenin-11-dUTP

Furthermore, when the dot-blot assay is carried out to test the labelling with digoxigenin, *Protocol 2* is used, but anti-digoxigenin antibody-conjugated alkaline phosphatase is used instead of streptavidin-conjugated alkaline phosphatase (step 6).

4. *In situ* hybridization and detection

4.1 Standard protocol for *in situ* hybridization

This procedure, comprising *Protocols 3–6*, is used when all the sequences of a probe should contribute to the hybridization signal, such as chromosome specific repetitive DNA probes, unique c-DNAs or genomic DNA fragments without IRS sequences. In the following the protocol is given for a 10 μl hybridization volume. This can be conveniently used when a 18 mm^2 coverslip is applied to cover the hybridization mixture on the slide. When larger areas are to be hybridized, scale up the solutions accordingly.

4.1.1 Probe mixture and probe denaturation

When very small volumes of DNA solutions (1 or 2 μl) are added, the DNA can be lyophilized before resuspension in formamide. With increasing volumes of DNA solution, we usually get less background in the hybridizations when precipitating the DNA before resuspension in formamide.

Protocol 3. Probe mixture and probe denaturation

Materials

- 3 M sodium acetate pH 5.0.
- Deionize formamide (several batches from several sources should be tested) using ion exchange resin (e.g Dowex XG8).
- Hybridization buffer containing 4 × SSC and 20% dextran sulphate. Prepare 20 × SSC and 50% dextran sulphate solutions. Dissolve dextran sulphate thoroughly, then autoclave it or filter it through a nitrocellulose filter. Combine 200 μl of 20 × SSC and 400 μl of 50% dextran sulphate with 400 μl of double-distilled water. Store at 4°C. Pipette carefully because dextran sulphate is very viscous.

Protocol 3. *Continued*

Method

1. Lyophilize 10–20 ng of labelled probe of a chromosome specific repeat cluster.

   or

   precipitate 20–60 ng of labelled single-copy DNA[a]. Add 1/20th vol. of 3 M sodium acetate and 2 vol. of ethanol, mix well, incubate at −70°C for 30 min, spin in Eppendorf centrifuge for 15 min; discard the supernatant (in most cases the small pellets are visible); wash the pellet by adding 400 μl 70% ethanol; spin for 5 min; discard the supernatant; lyophilize.
2. Resuspend in 5 μl of deionized formamide by vortexing or vigorously shaking for several minutes. Note that biotinylated DNA dissolves better in formamide than in water.
3. Add 5 μl of hybridization buffer and mix well. During this step, proceed with chromosome denaturation (see *Protocol 4*).
4. Denature the probe DNA by incubation at 75°C for 5 min followed by chilling on ice for approximately 5 min. Apply to denatured chromosomes (see *Protocol 4*).

[a] As an option 5 μg of sheared salmon sperm DNA can be added as carrier to any of these probes and could be co-lyophilized or co-precipitated with the probe.

Protocol 4. Chromosome denaturation

Materials

- Denaturation solution containing 70% deionized formamide and 2 × SSC. Check the pH value and adjust it to approximately pH 7.0, if necessary.
- 70% ice-cold ethanol.
- 90% ice-cold ethanol.
- 100% ice-cold ethanol.

Method

1. Label the area to be hybridized on the slide by scratching on the under side of the slide with a diamond pen.
2. Pre-warm the slides to 50–60°C in an incubator in order to prevent dropping of the temperature when the slides are put into the denaturation solution.

3. Add denaturation solution into a Coplin jar and heat it in a water bath to 70°C. Check temperature with a thermometer inside (!) the Coplin jar (the temperature should not drop below 68°C).
4. Put pre-warmed slides into the denaturation solution and incubate for *exactly* 2 min.
5. Transfer the slides to a Coplin jar (on ice) with cold 70% ethanol; incubate for 3 min.
6. Transfer the slides to a Coplin jar (on ice) with cold 90% ethanol; incubate for 3 min.
7. Transfer the slides to a Coplin jar (on ice) with cold 100% ethanol; incubate for 3 min.
8. Air-dry the slides.

When chromosomes are over-denatured, they usually look fuzzy when counterstained with propidium iodide or DAPI. Very bright staining with these dyes might be an indication of insufficient chromosomal denaturation.

Protocol 5. Hybridization

Method

1. Add 10 µl hybridization mixture with denatured probe to denatured chromosome preparation.
2. Put an 18 mm^2 coverslip on the hybridization mixture droplet. Avoid air bubbles!
3. Seal the edges of the coverslip with rubber cement.
4. Transfer the slides into a wet chamber (lidded dish with wet paper towels at the bottom and a frame to put slides on) and incubate at 37°C overnight.

Protocol 6. Detection

During the whole procedure the slides should never get dry.

Materials

- Washing solution A containing 50% formamide (does not need to be deionized) and 2 × SSC, pre-warmed to 42°C.

Protocol 6. *Continued*

- Washing solution B consisting of 1 × SSC (this concentration can vary according to the stringency for a particular probe; see below), pre-warmed to 60°C.
- Blocking solution containing 4 × SSC and 3% BSA (fraction V). Alternatively, dry fat milk (supplied as powder) can be used instead of BSA.
- Detection cocktail containing 4 × SSC, 1% BSA, and 0.1% Tween 20.
- Washing solution C containing 4 × SSC and 0.1% Tween 20, pre-warmed to 42°C.
- Counterstaining solution containing 2 × SSC and 200 ng/ml propidium iodide, or 200 ng/ml DAPI, or 200 ng/ml of both counter-stains.[a]
- Washing solution D containing 2 × SSC and 0.05% Tween 20.
- Antifade-mounting medium. Dissolve 0.233g 1,4-diazobicyclo (2.2.2) octane (DABCO) in 800 μl double-distilled water, add 200 μl 1 M Tris–HCl pH 8.0, add 9 ml glycerol and mix by inverting. Store in the dark at 4°C. By applying this solution the bleaching of fluorochromes after excitation is considerably decreased (41).

Method

1. Take the slides out of the wet chamber and carefully remove the rubber cement using forceps.
2. Transfer the slides into a Coplin jar containing washing solution A pre-warmed to 42°C, agitate in shaking water bath for 10–15 min (until the coverslips slide off).
3. Transfer the slides, without coverslips, into a new Coplin jar with washing solution A and agitate for 5 min.
4. Change the pre-warmed washing solution A twice more and shake for 5 min each.
5. Change three times into pre-warmed (60°C) washing solution B and shake for 5 min each.
6. Take the slides out of the Coplin jar, drain them as efficiently as possible by absorbing solution from the slide edges with a paper towel, and add 200 μl blocking solution. Cover with a 22 × 40 mm coverslip.
7. Transfer the slides into a wet chamber and incubate for 30 min at 37°C.
8. Lift up each slide and let the coverslip slide off, drain excess fluid as described above, and add 200 μl detection cocktail containing fluorochrome-conjugated detection reagent.[b] Cover with a 22 × 40 mm coverslip.[c]

   Higher signal intensities of digoxigenin labelled probe can be achieved by an indirect immunofluorescence detection (42). In this case substitute step 8 by the following procedure:

i. Lift up the slide to let the coverslip slide off, drain it as thoroughly as possible by absorbing solution from the slide edges with a paper towel, and add 200 µl detection cocktail containing 3 µg/ml sheep anti-digoxigenin antibody.

ii. Lift up the slide to let the coverslip slide off, and put it in a Coplin jar with pre-warmed washing solution C. Agitate at 42°C for 5 min.

iii. Change the washing solution twice more and shake for 5 min at 42°C.

iv. Take each slide out of the Coplin jar, drain it as described above and add 200 µl detection cocktail containing 3 µg/ml fluorochrome-conjugated anti-sheep antibodies. Cover with a 22 × 40 mm coverslip.

9. Transfer the slides into a wet chamber and incubate for 30 min at 37°C.

10. Lift up each slide and let the coverslip slide off, and put it in a Coplin jar with pre-warmed washing solution C. Agitate at 42°C for 5 min.

11. Change the washing solution twice more and shake for 5 min at 42°C.

12. Put into a Coplin jar (wrapped with aluminium foil) containing counterstaining solution and agitate at room temperature for 15 min.

13. Exchange counterstaining solution with washing solution D and incubate for 1 min (room temperature).

14. Take each slide out of the Coplin jar, drain excess fluid with a paper towel, and add 30 µl anti-fade solution. Cover with a 22 × 40 mm coverslip, avoiding air bubbles. Drain the edges of the coverslip and put the slide into a dark container. Short-term storage is carried out at room temperature and long-term storage at 4°C.[d]

[a] When rhodamine or Texas red is used for probe detection propidium iodide cannot be used as counterstain due to overlapping emission spectra of the fluorochromes. For the same reason AMCA and DAPI cannot be combined. In standard reactions, we detect a probe with FITC and counterstain with propidium and DAPI simultaneously (see also Section 5).

[b] For probe detection of biotinylated probe usually 5 µg/ml avidin-FITC is used. However, when different fluorochromes are to be applied, the same concentration of avidin–rhodamine, avidin–AMCA, etc., can be used. For detection of digoxigenin labelled probe 3 µg/ml of FITC-conjugated (or rhodamine-conjugated) anti-digoxigenin antibody is used. When biotin and digoxigenin labelled probes were hybridized simultaneously, both detection reagents, e.g. avidin–rhodamine and FITC-conjugated anti-digoxigenin, can be added simultaneously to the detection cocktail.

[c] Keep slides in the dark as much as possible after fluorochrome reagents have been applied.

[d] After microscopic inspection, the slides can be subjected to a signal amplification procedure (see Section 4.3).

In order to obtain specific signals from sequences with homologies to other chromosomal regions, it might be necessary to vary the stringency of the procedure. This is especially true for chromosome specific alphoid DNA repeats. Practically, the stringency can be increased best by decreasing the

salt concentration (e.g. 2 × SSC instead of 4 × SSC in the hybridization buffer in *Protocol 3*) or increasing the formamide concentration (e.g. from 50 to 70%) during the *in situ* hybridization. Furthermore, the post-hybridization washes (*Protocol 6*, step 5, can be carried out at higher stringencies by using lower salt concentrations (0.3 × SSC or even 0.1 × SSC as washing solution B).

4.1.2 Detection by a colorimetric assay

Commercial kits are available for a number of detection assays using alkaline phosphatase or horse-radish peroxidase conjugated to the detection reagent (avidin or antibodies). For visualization various substrates for the enzymes are used, which are converted by the enzymatic activity to products forming coloured precipitates. The procedures are very similar for the various combinations of enzymes and substrates. *Note*: most substrates of these enzymes, including 3,3-diaminobenzidine-tetrahydrochloride (DAB), are hazardous; use gloves and take care of the waste material. A protocol for using horse-radish peroxidase as enzyme and DAB as substrate is given below. The procedure corresponds to the above detection procedure (*Protocol 6*) until step 6.

Protocol 7. Detection by a colorimetric assay

Materials

- Blocking solution containing phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.5 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.0) and 3% BSA (fraction V).
- Detection solution containing PBS, 1% BSA and 0.1% Tween 20.
- Washing solution containing PBS and 0.1% Tween 20, pre-warmed to 42°C.
- DAB/H_2O_2 staining solution. Combine 3 ml PBS, 30 µl DAB stock solution (50 mg DAB per millilitre of water), and 15 µl H_2O_2 immediately before use.

Method

1. Perform post-hybridization washes as described in *Protocol 6*, steps 1 to 5.
2. Take the slides out of the Coplin jar, drain as efficiently as possible by absorbing solution from the slide edges with a paper towel, and add 200 µl blocking solution. Cover with a 22 × 40 mm coverslip.
3. Transfer the slides into a wet chamber and incubate for 30 min at 37°C.
4. Lift up each slide and let the coverslip slide off, drain excess fluid as

described above, and add 200 µl detection solution containing 5 µg/ml of streptavidin. Cover with a 22 × 40 mm coverslip.[a]

5. Transfer the slides into a wet chamber and incubate for 30 min at 37°C.
6. Lift up each slide and let the coverslip slide off, and put it in a Coplin jar with pre-warmed washing solution. Agitate at 42°C for 5 min.
7. Change the washing solution twice more and shake for 5 min at 42°C.
8. Take each slide out of the Coplin jar, drain excess fluid as described above, and add 200 µl detection solution containing 3 µg/ml biotinylated horse-radish peroxidase. Cover with a 22 × 40 mm coverslip.
9. Transfer the slides into a wet chamber and incubate for 30 min at 37°C.
10. Lift up each slide and let the coverslip slide off, and put it in a Coplin jar with pre-warmed washing solution. Agitate at 42°C for 5 min.
11. Change the washing solution twice more and shake for 5 min at 42°C.
12. Incubate in the dark with DAB/H_2O_2 staining solution for 10–20 min, or until sufficient substrate precipitate has accumulated, at room temperature.
13. Wash briefly in PBS, counterstain if necessary and mount as described in *Protocol 6*.
14. Evaluate the precipitated DAB product by transmission light microscopy.[b]

[a] Note that some suppliers offer a complex of streptavidin and biotinylated horse-radish peroxidase. In this case steps 6 to 9 are omitted. However, this complex may penetrate insufficiently into nuclei.

[b] For combination with chromosome banding (*Figure 7*) see Section 5.3.

4.2 Chromosomal *in situ* suppression (CISS) hybridization

This protocol is used when genomic DNA fragments (generally all genomic probes) or cDNAs containing IRS sequences are used as probe. The general difference to the standard protocol is the combination of probe and competitor DNA and an additional pre-annealing step after probe denaturation. Again the protocol is given for a 10 µl hybridization volume.

Depending on the complexity of the probe or target sequences, different concentrations of labelled probe are used. The following guide-lines have proved to be useful for determining the probe amount. Amounts below are given for 10 µl hybridization volumes:

- Single cosmids, plasmids, or phages 20–60 ng
- Band specific probe pools 100–200 ng
- Gel-purified single YACs 50 ng
- Total yeast DNA containing a YAC 1 µg

- Isolated inserts of a library derived from individual sorted human chromosomes 50–250 ng
  - e.g. F- and G-group chromosomes 50 ng
    C-group chromosomes 150–200 ng
    A-group chromosomes 250 ng
- Total DNA from a library derived from individual sorted human chromosomes
  - when vector to insert ratio is ~1:1 150–500 ng
    e.g. F- and G-group chromosomes 150 ng
    C-group chromosomes 300–350 ng
    A-group chromosomes 500 ng
  - when vector to insert ratio is ~10:1 0.5–2 μg
    e.g. F- and G-group chromosomes 0.5 μg
    C-group chromosomes 1–1.5 μg
    A-group chromosomes 2 μg

Protocol 8. Chromosomal *in situ* suppression hybridization

Materials

- 1 μg/μl salmon sperm DNA sheared or DNase digested to a size of approximately 500 nt (commercially available).
- 1 μg/μl human placental DNA digested with DNase to a size of 200–500 nt (digestion as described in *Protocol 1*, or 1 μg/μl *Cot1* fraction of human DNA with the same size range (commercially available)).
- Solutions to be prepared for the *Protocols 3–6*.
- Labelled probe as above.

Method

1. Combine the labelled probe DNA with 1–3 μg human competitor DNA (total human or *Cot1* fraction) and add salmon sperm DNA to adjust to a total amount of 10 μg DNA.
2. Add 1/20th vol. of 3 M sodium acetate and 2 vol. of ethanol, mix well, incubate at −70°C for 30 min, spin in Eppendorf centrifuge for 15 min, discard supernatant, wash the pellet by adding 400 μl 70% ethanol, spin for 5 min, discard the supernatant, and lyophilize.
3. Resuspend the pellet (which is visible in most cases) in 5 μl of deionized formamide by vortexing or vigorously shaking for several minutes.
4. Add 5 μl of hybridization buffer (*Protocol 3*) and mix well. During this step proceed with chromosome denaturation (see *Protocol 4*).
5. Denature the DNA by incubation at 75°C for 5 min. During this step start

pre-warming the slides with the denatured chromosomes (see *Protocol 4*) to 42°C (heat a plate or use a metal tube in a water bath).

6. Transfer the tubes from 75°C quickly to 37°.
7. Incubate for 5–15 min to allow pre-annealing.
8. Apply the pre-annealed DNA solution to denatured chromosomes on pre-warmed slides.
9. Continue as described in *Protocols 5* and *6*.

4.3 Signal amplification

Some laboratories routinely use signal amplification procedures in order to detect fluorescence signals. The principle of this method is illustrated in *Figure 4*. The technique is based on binding of antibodies directed against the previously used detection reagent (avidin or antibody). The procedure can be either direct (left half of *Figure 4*) using one layer of fluorescently labelled antibodies, or indirect (right half of *Figure 4*) using one layer of antibodies conjugated with a reporter group and a second layer of fluorescent reagents directed against the reporter groups. In case of indirect amplification, usually the last detection reagent is the same as the one used for the primary detection; this procedure is therefore also referred to as sandwich amplification. It allows several cycles of amplification by using only two different reagents. Occasionally, we apply one cycle of sandwich amplification when probes are very small, resulting in weak signals. However, the amplification is not linear: while the signal intensity is increasing, the signal-to-noise ratio is decreasing. We recommend that signal amplification is performed only when slides are low in background. The protocol below, designed for the signal amplification of biotin labelled probes, is based on the work by Pinkel *et al.* (14).

Protocol 9. Signal amplification

Materials

- Washing solution C containing 4 × SSC and 0.1% Tween 20, pre-warmed to 42°C.
- Detection cocktail containing 4 × SSC, 1% BSA, and 0.1% Tween 20.

Method

1. Carefully remove the coverslip from the mounted slide (see Section 4.1).
2. Put the slides in a Coplin jar containing washing solution C, which is pre-warmed in a 42°C shaking water bath. Gently agitate for 10 min.
3. Change the washing solution C three more times and shake in 42°C for 10 min each.

Protocol 9. *Continued*

4. Take the slide out of the Coplin jar, drain it as thoroughly as possible by absorbing solution from the slide edges with a paper towel, and add 200 µl detection cocktail containing 1–5 µg/ml biotinylated anti-avidin antibodies. Cover with a 22 × 40 mm coverslip.
5. Transfer the slide into a wet chamber and incubate for 30 min at 37°C.
6. Lift up the slide to let the coverslip slide off, and put it in a Coplin jar with pre-warmed washing solution C. Agitate at 42°C for 5 min.
7. Change the washing solution twice more and shake for 5 min at 42°C.
8. Take the slide out of the Coplin jar, drain it as described above, and add 200 µl detection cocktail containing 5 µg/ml avidin-conjugated fluorochrome (e.g. avidin–FITC). Cover with a 22 × 40 mm coverslip.
9. Repeat washing as before, see steps 6 and 7.
10. Counterstain chromosomes and mount as described above (*Protocol 6*).

5. Chromosome counterstaining and banding

The rapid and precise relative mapping of fluorescently labelled probes along counterstained chromosomes has been demonstrated (42). By a combination of *in situ* hybridization to prometaphase chromosomes with digital imaging microscopy (see Section 6) numerous probes can be ordered using mapping coordinates defined in the following way: the distance between the signal and a reference point is given as the fraction of the length of the total chromosome or a sub-chromosomal region. For example, an 'FLpter' value is the fractional length of a chromosome with reference point pter. It should be noted that the ISCN banding ideograms (43) are not normalized relative to the fractional length of chromosomes. Therefore, mapping coordinates defined by fractional length values cannot be extrapolated from the ISCN ideograms in order to define them as a band locus. However, this approach is very useful to map the relative chromosomal position of multiple probes (for further discussion see ref. 8).

To verify a chromosome assignment, co-hybridization with a probe or probe set known to map to the target chromosome of interest can be easily performed (preferably by a dual colour experiment). In order to express mapping data in terms of classical cytogenetic terminology, banding methods can be combined with non-isotopic *in situ* hybridization protocols. Fluorescence banding with a fluorochrome that emits in a range different to the fluorochrome used for probe detection is advantageous since it does not require a relocation of chromosomes. Banding by quinacrine, Hoechst 33258 chromomycin A_3 or DAPI (see Chapter 4 and ref. 44) can easily be

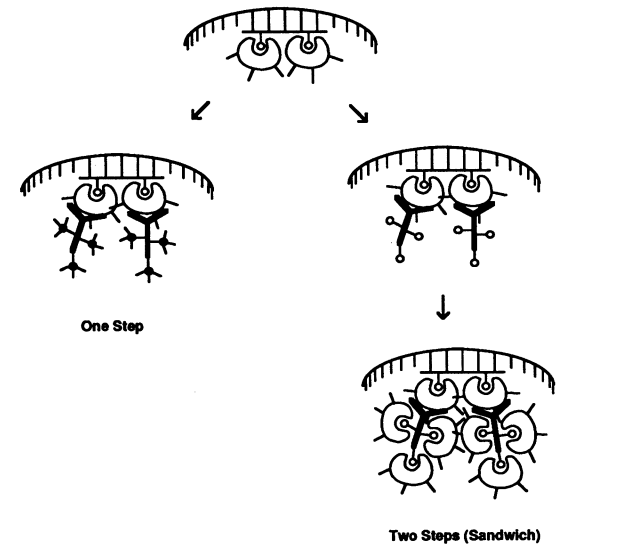


Figure 4. Schematic illustration of a signal amplification procedure. For further explanation see text.

performed after the hybridization/detection procedure (*Protocol 6*, step 6). On heat-denatured chromosomes, staining with DAPI alone, i.e. without additional stains such as actinomycin or distamycin, results in a differential Q-banding-like pattern of good quality (see *Figure 5*, panel B; see also ref. 45). However, differences in the staining pattern of heterochromatin by DAPI and quinacrine should be noted. Unfortunately, it is often difficult to document DAPI-banding by conventional photography. Due to the heat

denaturation of the chromosomes, a differential staining is also achieved with propidium iodide, resulting in an R-banding-like pattern. This banding is often pale (see *Figure 3,* A and C) and in our hands the quality has been very variable. Propidium iodide staining in combination with fluorescent replication banding (see *Protocol 11*), however, results in high quality banding (46, 47).

5.1 Alu-banding

Distinct chromosomal banding patterns can be produced by *in situ* hybridization with DNA probes for interspersed repetitive elements (IRS). Co-hybridization of a DNA probe with IRS sequence(s) causes a simultaneous *in situ* hybridization banding profile (42, 48). For human chromosomes Alu repeats are very useful, generating an R-banding like banding pattern. Both cloned Alu sequences and sequences generated by IRS-PCR (see Section 7) have been reported as co-hybridization probes (42, 49). An example of a cosmid mapping in combination with Alu-banding is shown in *Figure 5*, panels A and C.

Protocol 10. Alu-banding

1. Prepare a 10-μl probe mixture and perform probe denaturation as described in *Protocol 3* (without competition) or *Protocol 8* (with competition), depending on the characteristics of the probe. When CISS hybridization is performed, use only 1 μg competitor DNA per 10 μl volume.
2. Prepare a 5 μl probe mixture with 100–300 ng Alu-enriched DNA (e.g. a cloned fragment containing Alu elements; see ref. 42) and perform denaturation as described in *Protocol 3*.
3. Combine mixtures from steps 1 and 2 prior to application on to slides with denatured chromosomes (*Protocol 4*).
4. Proceed as described in *Protocol 5*.

5.2 GTG-banding

Well-established banding techniques may be preferred. These techniques can be performed with fluorescence *in situ* hybridization prior (pre-banding) or after *in situ* hybridization (post-banding). In experienced hands, both approaches give excellent results. Pre-banding has the advantage that any interference of chromosomal heat denaturation with banding quality can be avoided. A disadvantage is the fact that metaphase spreads have to be relocated and photographed twice. For pre-banding we have followed the protocol described by Klever *et al.* (50). Two examples are shown in *Figure 6*. After routine trypsin–Giemsa (GTG) banding (see Chapter 4) the preparations are treated as shown in *Protocol 11*.

Protocol 11. De-staining and post-fixation of GTG-banded chromosomes

1. Embed the preparations in Eukitt.
2. Locate metaphases and take photographs.
3. Remove the embedding material and Giemsa stain by the following washes in Coplin jars:
   - xylol for 1 min
   - xylol/ethanol (1:1, v/v) for 1 min
   - methanol/acetic acid (3:1, v/v), two times for 5 min
4. Air-dry the slides.
5. Transfer the slides in a Coplin jar containing 3.7% formaldehyde in phosphate-buffered saline (PBS, pH 7), incubate for 10 min.
6. Wash in PBS, twice more for 5 min.
7. Air-dry again.
8. Proceed as described in *Protocol 4*.

The additional fixation results in improved preservation of chromosomal morphology during heat denaturation.

5.3 Replication banding

For post-banding we can recommend replication banding procedures performed after 5-bromo-2'-deoxyuridine (BrdU) incorporation during S-phase. For details see refs 51 and 52.

Protocol 12. Fluorescent replication banding

1. Add BrdU and fluorodeoxyuridine (FdU) in a final concentration of 10 μg/ml and 0.5 μg/ml, respectively, to PHA-stimulated lymphocyte cultures from peripheral blood (see Chapter 2) after 65 h of cultivation.
2. Six hours later add colcemid to a final concentration of 0.05 μg/ml and cultivate cells for 30 min more.
3. Prepare metaphase spreads (see Chapter 2) and proceed with *in situ* hybridization as described above.
4. In the case of fluorescence *in situ* hybridization, the incorporated BrdU can be detected by simultaneous indirect immunofluorescence using monoclonal mouse-anti-BrdU antibodies for the first step and FITC- or TRITC-conjugated anti-mouse IgG antibodies for the second step. For this, proceed as described in *Protocol 6* (*indirect immunofluorescence* described under step 8 *i* to *iv*) with antibody concentrations according to the recommendations of the suppliers.

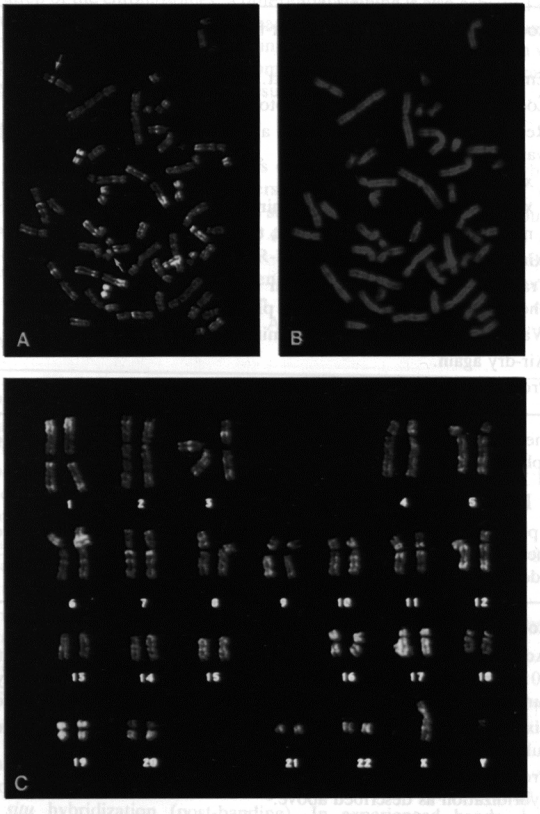


Figure 5. Simultaneous visualization of fluorescently labelled cosmid probe and Alu-banding. (A) Digoxigenin-labelled cosmid probe J1-2 (see ref. 42) and a biotin-labelled DNA probe containing four Alu-repeats were cohybridized and detected via rhodamine and FITC, respectively. Note the R-banding like pattern of the Alu probe signal. Chromosome identification and band assignment can easily be performed by using the Alu-banding. Cosmid J1-2 had been mapped by determining the fractional distance of the chromosome length (see text) to a chromosome region corresponding to 11p11.2. This is indeed the cytological map position, as the signal is within the brightly stained band 11p11.2 (see arrows). (B) DAPI-stain of the metaphase shown in A. Note, that the DAPI

If permanent staining is preferred, the Hoechst 33258–Giemsa technique can be applied to BrdU pulsed chromosome complements (53). This procedure has been used for isotopic techniques as well as non-isotopic techniques using permanent alkaline phosphatase or peroxidase (see Section 4.1.2) signal detection procedures (54, 55). Examples are shown in *Figure 7*. For a good banding quality it is important to adjust the pH of the solution used for heat denaturation of the chromosomes (see *Protocol 4*) to a range between pH 6 and pH 7, since a higher pH (>8) may result in massive swelling of the chromosomes (A. Brückner, unpublished observations). After signal detection, the slides are treated as described in *Protocol 13*.

Protocol 13. Permanent replication banding

Materials

- 33258 Hoechst-staining solution. Dissolve 5 mg of 33258 Hoechst in 100 ml 'Latt' buffer containing 0.15 M NaCl, 0.03 M KCl, 0.01 M phosphate (pH 7). Keep this stock solution in the dark at 4°C. Dilute in Latt buffer 1:200 just prior to use (final concentration 0.25 μg/ml).
- Giemsa solution. Combine 30 ml 67 mM KH_2PO_4, 30 ml 67 mM Na_2HPO_4, and 3 ml Gurr's–Giemsa stain R66.

Method

1. Perform steps 1–3 as described in *Protocol 12*.
2. Incubate the slides in a Coplin jar in the dark with 33258 Hoechst staining solution at room temperature for 15 min.
3. Briefly wash with water.
4. Put the slides into a dish, cover them with a thin layer of 2 × SSC just sufficient to prevent air-drying, and expose them to the light of a UV-mercury lamp.[a]
5. Incubate in 2 × SSC at 60°C for 90 min.

Figure 5 ***cont.***
stain produces a Q-banding-like pattern in heat denatured chromosomes, even without further treatment (such as actinomycin). Whereas Alu-probes do not label centromeres and several pericentromeric regions, the DAPI-counterstain reveals the full chromosome continuum (compare panels A and B). (C) Complete karyotype of the Alu-banded metaphase in panel C (46,XY). Note, that the quality of the Alu-banding is superior to the DAPI-banding.

Protocol 13. *Continued*

6. Stain the slide with Giemsa solution for approximately 20 min or until a good differentiation is visible.
7. Air-dry and embed in Eukitt.

[a] For example, the authors use a 30 W lamp at a distance of 20 cm for 30 min.

6. Microscopy

Signals from painted whole chromosomes, stained sub-chromosomal regions, or localized single probes are generally visible when an epifluorescence microscope is used. Examples of photographs taken on a standard microscope are shown in *Figure 6*. Counting signals in interphase nuclei can easily be carried out with a standard equipment. Potential users who are inexperienced in the field of fluorescence microscopy should seek expert advice before they buy their own system. It is very important to have appropriate objectives and filter sets for the visualization of the essential fluorochromes. The most commonly used fluorochromes are (a) blue colour: DAPI (chromosome staining) and AMCA (conjugated to avidin, antibody, or nucleotides); (b) green colour: quinacrine (chromosome staining), FITC or bidopy (both conjugated); (c) red colour: propidium iodide (chromosome staining), rhodamine or Texas red (both conjugated). Filter sets for various fluorochromes are described elsewhere in this book (see Chapter 8). For some applications a filter set may be used which is very selective for a given fluorochrome, while in other applications filter sets may be used which allow the simultaneous detection of two fluorochromes, such as FITC for signal detection and propidium iodide for chromosome-staining (see *Figure 3*). Double band-pass filter sets have become available, which allow the simultaneous recording of fluorochromes, such as FITC and rhodamine. Such filter sets may become very important in two colour mapping experiments, when the geometric relation of two distinctly coloured sites have to be recorded as accurately as possible. A change of filter sets generally leads to more or less pronounced shifts, detectable for example after double exposure of photographic films (registration problem).

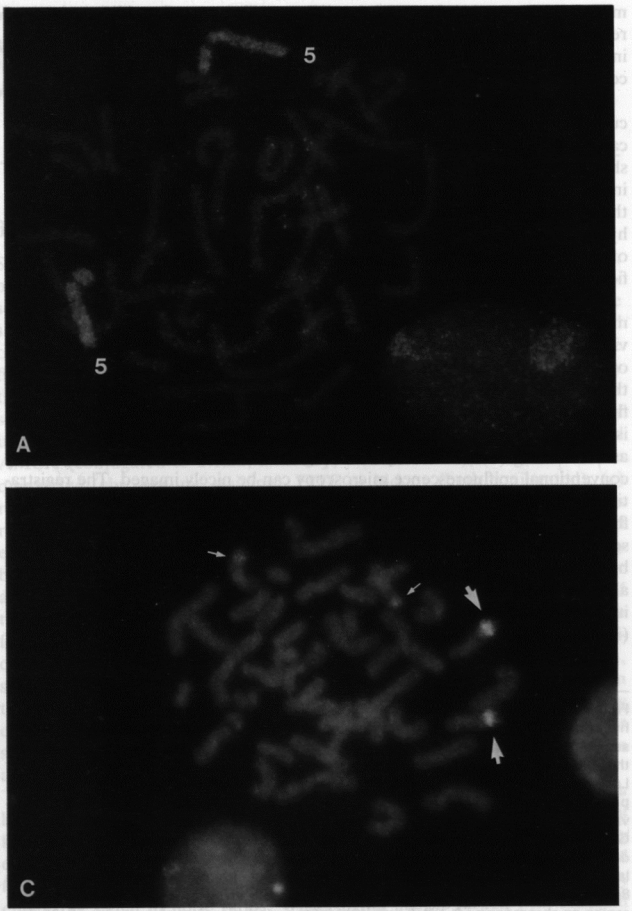
In order to exploit the full potential of multicolour fluorescence *in situ* hybridization in clinical and tumour cytogenetics, quantitative digital fluorescence microscopy is clearly needed. Digitized images are not only easier to handle but can also be subject to powerful image processing. Digital filtering procedures can facilitate the analysis considerably; for instance, by enhancing signal to noise ratios. When digital images (taken with different filter sets from the same object) are electronically overlayed, the registration problem is again evident. At present a perfect solution to this problem is not available. It is advisable to ask the commercial supplier for a special align-

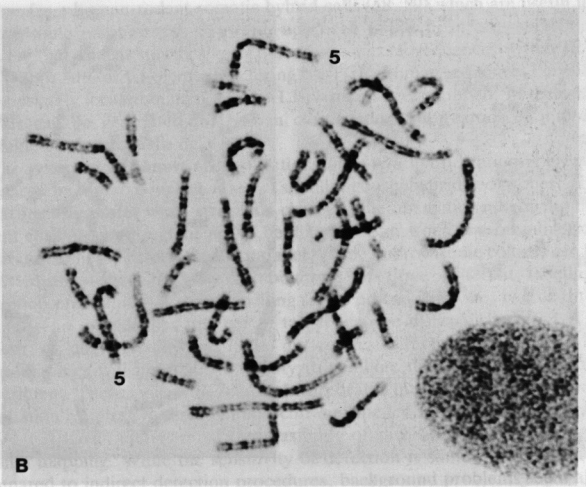
ment of the emission filters of the different filter sets. In addition, internal reference points may be included in a specimen by point-like particles carrying the various fluorescent dyes. This could greatly facilitate the automated correction of misalignments by appropriate software.

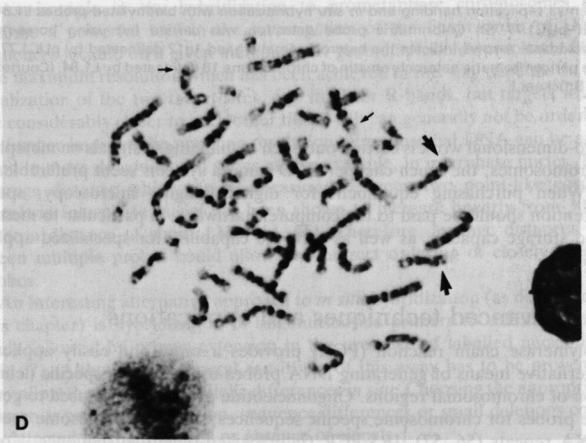
Different camera systems allow the generation of digitized images. The currently most sensitive system is the cooled CCD (charged coupled device) camera. Examples of images generated by using a cooled CCD camera are shown in *Figure 5*. This device allows photon counting, and with longer integration times fluorescence signals can be detected which are not visible to the observer's eyes. This might improve the analysis of fluorescence *in situ* hybridization with probes smaller than 2 kb (see Section 1). For the recording of chromosome painting images less expensive camera systems will be sufficient.

Digitized images can also be generated using a confocal laser scanning microscope. Examples are shown in *Figure 3*. In contrast to CCD or video-camera systems, the confocal laser microscope can greatly reduce the out-of-focus fluorescence allowing generation of high quality optical sections through the labelled specimen. Therefore, if three-dimensional recording of fluorescent cellular specimens is needed, a laser confocal scanning microscope is the instrument of choice. Although at present this device is not as sensitive as the current cooled CCD systems, signals visible to the observer's eyes by conventional epifluorescence microscopy can be nicely imaged. The registration problem can be overcome by parallel excitation/emission of different fluorochromes in the dual channel mode. However, using the confocal laser scanning microscope, one is limited to the use of the fluorochromes which can be excited by the lasers available in the instrument. Most instruments today allow excitation of one or two fluorochromes (green and red fluorochromes) in the standard set-up. Additional laser equipment for three colour detection (i.e. an additional UV-laser for blue fluorochromes) is very costly. When only

Figure 6. (Overleaf). Combination of CISS hybridization and GTG-banding (A) CISS-hybridization of biotinylated human chromosome 5 specific bacteriophage DNA library sequences to a fibroblast metaphase spread from *Macaca fuscata*. The probe delineates the entire homologue macaque chromosome except for the centromeric heterochromatin. Lack of labelling may be due to rapid divergence in heterochromatic sequences during primate evolution (J. Wienberg, A. Jauch, R. Stanyon, and T. Cremer, unpublished observation). Note the visualization of the two chromosome 5 domains in the cell nucleus. (B) GTG banding of the *Macaca fuscata* metaphase spread shown in (A). (C) FITC-signals detected on a human lymphocyte metaphase spread after CISS-hybridization of biotinylated microlibrary DNA derived from 37 microdissected human chromosomes 8 (bands 8q23-8q24.1) kindly provided by B. Horsthemke. Big arrows indicate the labelled subregion on chromosome 8, small arrows point to a weak signal on chromosome 9q33. This minor signal could be explained by an occasional error made during the microdissection procedure. (Courtesy of C. Lengauer; for details see ref. 32). (D) GTG-banding of the metaphase spread shown in (C).


5
5
A
C


5
5
B


D

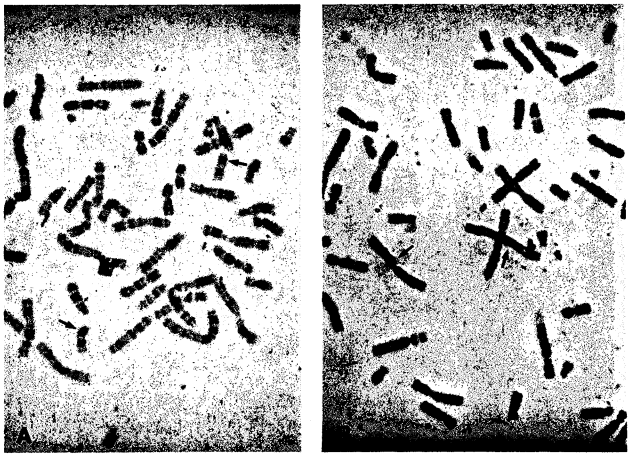


Figure 7. Combination of non-isotopic *in situ* hybridization and permanent replication banding. Male human lymphocyte metaphase chromosomes after Hoechst 33258–Giemsa replication banding and *in situ* hybridization with biotinylated probes L1.84 (A) and pUC1.77 (B) Colorimetric probe detection was carried out using horse-radish peroxidase. Arrows indicate the heterochromatic band 1q12 delineated by pUC1.77 and the pericentromeric heterochromatin of chromosome 18 delineated by L1.84. (Courtesy of A. Brückner.)

two-dimensional work is carried out, such as mapping of signals on metaphase chromosomes, the much cheaper CCD camera systems seem preferable.

When purchasing equipment for digital imaging microscopy, special attention should be paid to the computer hardware, in particular to memory and storage capacity, as well as software capability for specialized applications.

7. Advanced techniques and applications

Polymerase chain reaction (PCR) provides a rapid and easily applicable alternative means of generating DNA probes useful for the specific delineation of chromosomal regions. Oligonucleotide primers can be used to generate probes for chromosome specific sequences, such as chromosome specific DNA repeats (56, 57). IRS-PCR (interspersed-repetitive sequence – polymerase chain reaction) protocols have been applied to generate human DNA

probes from human-rodent somatic hybrid cells (58, 59) which are useful for chromosome painting (60, 61). This approach is based on oligonucleotide primers that anneal specifically to human specific subsequences within IRS, e.g. within Alu- or L1-elements. During the PCR, human sequences between appropriately located human Alu- or L1-elements are amplified. In this way, probes can be generated for human chromosome sub-regions of interest contained in hybrid cells or YACs.

The potential of almost all applications of *in situ* hybridization is greatly enhanced by multicolour detection of simultaneously hybridized probes. This is particularly useful when structural chromosome aberrations involving different chromosomal regions are to be diagnosed, or when several numerical aberrations should be detected in parallel. Three fluorochrome colours can be easily distinguished, and by combinations of three different labelling/detection procedures—i.e. by labelling single probes with one, two or three colours—an even higher number of targets can be distinguished (62). The advent of commercially available nucleotides directly conjugated with fluorochromes will improve the ability to combine multiple colours in one experiment. These nucleotides can be incorporated into DNA probes by nick-translation resulting in directly fluorochrome labelled DNA probes. Wiegant *et al.* (63) have demonstrated the feasibility of such an approach for rapid cosmid mapping. While the sensitivity of detection is somewhat reduced as compared to indirect detection procedures, background problems seem also considerably reduced.

Non-isotopic *in situ* hybridization to prometaphase chromosomes has become a powerful tool in the investigation of the linear order of DNA sequences located on a given metaphase or prometaphase chromosome (42). The maximum resolution which has been achieved in this way depends on the localization of the two target sites, e.g. in G- or R-bands, but targets which are considerably closer to each other than 1 Mb can generally not be ordered. To further improve the mapping resolution, chromosomal DNA can be analysed in more de-condensed states as, for example, in interphase nuclei. The average distance which can be measured between two point-like cosmid signals in interphase nuclei has been shown to increase linearly from 30 kb up to a distance of about 1 Mb (64, 65). Therefore, average distances between multiple probes could allow the indirect ordering of closely spaced probes.

An interesting alternative approach to *in situ* hybridization (as described in this chapter) is hybridization of oligonucleotide primers to cellular nucleic acids followed by primer extension in the presence of labelled nucleotides (66, 67). Although the general sensitivity of this assay has to be proven, it may allow the detection of allelic differences *in situ*. Choosing the appropriate primer or primer combination, sequence differences or small deletions could be detected by the presence or absence of signals.

Acknowledgements

We thank our colleagues Andreas Brückner, Anna Jauch, Johannes Wienberg, and Christoph Lengauer who have contributed unpublished material for illustrations to this chapter. We are grateful to David Ward for stimulating discussions.

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