

## Cytogenetic and FISH Techniques in Myeloid Malignancies

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### Summary

Chromosome analysis is an essential part of the diagnostic testing of myeloid malignancies. Good chromosome preparations are essential for a complete cytogenetic analysis. This means plentiful metaphase spreads with well-spread crisply banded chromosomes. To achieve such a result, several variables, including the growth rate of the leukemic cells, are critical. The method described in this chapter has been extensively tested and should produce reasonable results from most cases. Fluorescence *in situ* hybridization (FISH) is less influenced by sample variation and as a result may be obtained from either metaphase spreads or interphase cells. Moreover, FISH is capable of describing chromosome rearrangements at the gene level, rather than at the gross level shown by conventional cytogenetics. It does not, however, provide information on genetic rearrangements other than at the specific target site of the probe used, unlike conventional cytogenetics. Thus, these two techniques complement each other and are both now essential elements of chromosome analysis.

**Key Words:** Cytogenetic; karyotype; synchronization; harvest; metaphase; banding; fluorescence *in situ* hybridization.

### 1. Introduction

Over the past 40 yr, cytogenetic analysis has become an integral part of the diagnosis and management of patients with myeloid malignancies. Cytogenetic analysis is required to diagnose disorders such as chronic myeloid leukemia (CML) and to provide information regarding prognosis in acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) (1–3). Although specific genetic targets can be detected using molecular techniques such as reverse-transcription polymerase chain reaction, cytogenetic analysis provides a global view of genetic rearrangements within the malignant cell. There are a number of different methods available for producing metaphase spreads suitable for cytogenetic analysis. The methods described below are in routine use in the Victorian Cancer Cytogenetics Service (VCCS). The number and type of cul-

tures established depends on the diagnosis, but good practice dictates the setting up, where possible, of at least two cultures for each sample. For most myeloid disorders, a culture using an agent to produce cell synchrony is preferred, and so if limited patient material enables the establishment of only one culture, a synchronized culture is recommended. With new acute leukemias, the subtype may not have been identified by the time the cultures are established, and so at the VCCS an overnight unsynchronized culture is established preferentially, and, if sample permits, a synchronized culture is set up as second choice. Synchronization requires a 48-h culture, and acute lymphoblastic leukemia cells are generally less capable than AML cells of surviving more than 24 h in culture.

*Cell synchronization* refers to a method of increasing the number of cells that have reached the metaphase part of the cell cycle, when harvesting of the culture is initiated. An agent is added to the culture to block DNA synthesis; the following method uses 5'-fluorodeoxyuridine (FdU) with added uridine. By blocking the cell cycle, a large number of cells are collected that have all arrived at the same point of division. Release of the blockage then allows them all to proceed through division together. FdU acts as an antagonist to thymidylate synthetase. BrdU (5-bromo-2'-deoxyuridine), an analog of thymidine, is then used to release the block. The harvest is timed for approx 7 h after release so that the maximum number of cells has arrived at metaphase (4). Metaphase is the stage of mitosis at which chromosomes are most contracted, prior to the chromatids separating to travel to opposite poles of the cell; they are most distinguishable one from another at this point.

Cells are harvested by the addition of Colcemid®, a synthetic analog of colchicine, which prevents the formation of the cell spindle fibers and so prevents the onset of anaphase. The cells are treated with a hypotonic saline solution to increase the cell volume and so allow the chromosomes to disentangle from each other. Finally, the cells are fixed in a mixture of methanol and glacial acetic acid. The fixed suspension is dropped onto glass slides, which are then air-dried and ready for either G-banding or fluorescence *in situ* hybridization (FISH) studies. G-banding stands for Giemsa banding, named after the German chemist Gustav Giemsa, but the characteristic banding pattern of dark and light bands along each chromosome can be induced using a number of different stains. The method described here uses Leishman's stain. Prior to G-banding, the slides must be "aged." When banding methods were first introduced, it was discovered that G-bands could be induced in chromosome preparations only after the slides had been allowed to sit for several days or even weeks. The pressures on clinical cytogenetics laboratories today require rather speedier turn-around times than this leisurely practice would allow. It has therefore been necessary to simulate aging. The method below describes

the heating of slides on a 100°C hotplate for a few minutes to “age” slides prior to immersing them in a weak trypsin solution and staining with Leishman’s stain to produce G-banding. After air-drying, the slides are then cover-slipped and are ready for cytogenetic analysis (5).

FISH is a method by which specific DNA sequences are labeled with a fluorescent tag and applied to metaphase chromosomes or interphase nuclei so that the DNA sequences or probes hybridize to their corresponding sequences within the target cell. The fluorescent tag indicates the presence and position of specific sequences within the cell. FISH may be performed on cytogenetic preparations, tissue imprints, bone marrow smears, or paraffin-embedded sections. FISH is a valuable complementary test to conventional cytogenetics. In cases of poor chromosome morphology, it is capable of accurately detecting subtle abnormalities that are of prognostic importance, such as the inversion 16 in acute myelomonocytic leukemia with abnormal eosinophils, and of detecting cryptic rearrangements and deletions such as the deletion that occurs at the *ABL/BCR* breakpoint in a proportion of CML cases (6,7). The method described here refers to FISH performed on cytogenetic suspension dropped onto glass slides.

To allow hybridization, target and probe are both denatured to single-strand DNA. This is achieved by heat and in the presence of formamide and salt solution (both components of the hybridization buffer). The incubation time required for hybridization varies from a few hours to overnight. The specificity of hybridization to target DNA can be controlled by variations of temperature, pH, formamide, and salt concentration in the hybridization buffer. Despite a number of mismatched bases along the strands of DNA, stable duplexes can form under certain hybridization conditions, leading to cross-hybridization or “background.” Under conditions of high stringency, only probes with high homology to the target sequence will form stable hybrids, resulting in low or no background hybridization. However, low-stringency conditions (reactions carried out at low temperature, high salt, or low formamide concentrations) may result in high background or nonspecific probe binding (8).

Once the excess probe has been removed in a series of post-hybridization washes, the fluorescent signals are detected via a fluorescence microscope. Scoring of signals and interpretation of results varies according to the probes used and the initial indication for FISH studies.

The following methods outline (1) the establishment of both overnight and synchronized cultures of bone marrow cells from patients with myeloid disorders, and the harvesting of cells from each culture after varying periods of incubation; (2) slide making and the production of banded metaphase spreads for microscopic analysis; and (3) FISH performed on metaphase spreads and interphase cells.

## 2. Materials

The materials required for each step have been listed in separate sections according to the stage at which they are required.

### 2.1. Bone Marrow Culture and Harvest

1. Bone marrow aspirate, 0.5–1.0 mL, collected in a sterile syringe or tube containing approx 100 U of preservative-free heparin.
2. RPMI 1640 medium with L-glutamine and HEPES (*see Note 1*).
3. Fetal calf (bovine) serum (FCS): stored at  $-20^{\circ}\text{C}$  in aliquots (i.e., 20-mL aliquots for addition to 200-mL bottles of medium to produce a 10% solution). This should be thawed and added to medium immediately before use.
4. L-Glutamine-penicillin-streptomycin (PSG): a mixture containing 200 mM L-glutamine, 10,000 U of penicillin, and 10 mg streptomycin per mL. PSG is stored at  $-20^{\circ}\text{C}$  and 1 mL is added to a 200-mL bottle of medium immediately prior to use.
5. Sterile 50-mL tissue-culture flasks.
6. Sterile pipets.
7. FdU (5'-fluorodeoxyuridine, MW = 246.2) and uridine (MW = 244.2) working solution: 1 mL uridine solution ( $4 \times 10^{-3} M$ ) and 1 mL FdU solution ( $5 \times 10^{-5} M$ ) added to 8 mL sterile dH<sub>2</sub>O to give working concentrations of  $5 \times 10^{-6} M$  FdU and  $4 \times 10^{-4} M$  uridine (stored at  $4^{\circ}\text{C}$ ).
8. 5-Bromo-2'-deoxyuridine (BrdU): a  $2 \times 10^{-3} M$  working solution (stored at  $4^{\circ}\text{C}$ ).
9. Colcemid: 10  $\mu\text{g/mL}$  solution.
10. Hypotonic solution (potassium chloride 0.075 M): a 5.59 g/L solution of KCl (MW = 74.55).
11. Carnoy's solution: 3:1 (v/v) analar methanol/glacial acetic acid, made fresh just before use.
12. 15-mL plastic, nonsterile screw-top centrifuge tubes.
13. Microscope glass slides: 76 mm  $\times$  26 mm superfrost slides with ground edges.

### 2.2. Banding

1. Trypsin: desiccated tryptic enzyme rehydrated in 10 mL sterile distilled H<sub>2</sub>O and aliquots stored at  $-20^{\circ}\text{C}$ . The amount of desiccated trypsin provided by the manufacturer is based on its enzyme activity rather than weight, and so the solution is prepared according to the manufacturer's instructions to produce a 5% solution of trypsin (1:250). A working solution is made fresh daily by adding 0.25 mL trypsin solution to 35 mL trypsin diluent (discussed later) and 35 mL distilled H<sub>2</sub>O in a Coplin jar.
2. Trypsin diluent: NaCl (8.0 g), KCl (0.4 g), Na<sub>2</sub>HPO<sub>4</sub> (0.06 g), KH<sub>2</sub>PO<sub>4</sub> (0.06 g), and NaHCO<sub>3</sub> (0.5 g) dissolved in 1 L distilled H<sub>2</sub>O and stored at  $4^{\circ}\text{C}$  prior to use.
3. Ca<sup>2+</sup>/Mg<sup>2+</sup> free solution: NaCl (8.0 g), KCl (0.2 g), Na<sub>2</sub>HPO<sub>4</sub> (1.15 g), and KH<sub>2</sub>PO<sub>4</sub> (0.2 g) dissolved in 1 L distilled H<sub>2</sub>O and stored at  $4^{\circ}\text{C}$  prior to use.
4. Leishman's solution (eosin methylene blue compound) (*see Note 2*) stored in powdered form at room temperature. Leishman's stain powder (2.8 g) is added to 1 L analytical-grade methanol and mixed with a magnetic stirrer for 3 h. The

resulting solution is incubated at 37°C for at least 1 wk. To prepare a working solution, 100 mL is filtered through Whatman No. 1 filter paper. The rest is stored in the dark at room temperature until required. The filtered Leishman's stain is then diluted 1 in 10 in stain buffer (discussed later) immediately prior to use.

5. Stain buffer: one Gurr® buffer tablet (pH 6.8) is dissolved in 1 L distilled H<sub>2</sub>O and stored at 4°C prior to use.
6. DPX mounting solution, stored at room temperature.
7. Glass cover slips: 24 mm × 50 mm in size.

### 2.3. FISH

1. Microscope glass slides: 76 mm × 26 mm superfrost slides with ground edges; slides should be prepared as for conventional cytogenetic analysis.
2. Diamond pen for marking glass slides.
3. Dry block heater for co-denaturation of target and probe DNA. This heater should be capable of accurate temperature control at 72–74°C.
4. Hotplate heated to 37°C.
5. Hybridization box: a shallow plastic box with a sealable lid, microwavable and able to accommodate several slides laid inside horizontally.
6. Piece of absorbent foam to sit inside the hybridization box.
7. Cover slip of size determined by the amount of probe solution used (*see Note 3*). In general, the 24 mm square cover slip is recommended. The volumes described below refer to use of this size cover slip.
8. Glue/rubber cement (*see Note 4*).
9. Premixed probe/hybridization buffer solution: the product information recommends a mixture of 1 µL probe/2 µL purified H<sub>2</sub>O/7 µL hybridization buffer (supplied with the probe and containing dextran sulphate, formamide, and SSC) (*see Note 5*).
10. 20X stock salt solution (SSC): 175.3 g NaCl and 88.2 g Na citrate dissolved in 1 L dH<sub>2</sub>O and the pH adjusted to 7 using 1 N HCl and 1 N NaOH; all SSC solutions may be stored at room temperature (but discarded if turbidity develops).
11. 4X SSC: 200 mL 20X SSC diluted in 800 mL dH<sub>2</sub>O and pH adjusted to 7.
12. 2X SSC: 100 mL 20X SSC diluted in 900 mL dH<sub>2</sub>O and pH adjusted to 7.
13. 0.4X SSC/0.3% NP40: 100 mL 4X SSC, 900 mL dH<sub>2</sub>O, and 3 mL NP40 combined in a glass bottle and stored at room temperature; inhalation of NP40 (polyethylene glycol octylphenyl ether) vapors should be avoided.
14. 2X SSC/0.1% NP40: 1 L 2X SSC and 1 mL NP40 combined in a 1-L sterile glass bottle and stored at room temperature.
15. DAPI counterstain: 20 µg/mL 4',6'-diamidino-2-phenylindole (DAPI) stock solution prepared.
16. DAPI/anti-fade working solution (*p*-phenylene diamine dihydrochloride): 100 mg *p*-phenylene diamine dihydrochloride (powder stored in a tightly sealed light-proof container at room temperature) added to 10 mL PBS and the pH adjusted to 8.0 with 0.5 M carbonate-bicarbonate buffer. The solution should have a slight pink tinge (if the color turns yellow/orange, the solution should be discarded).

The solution is filtered using a 0.22- $\mu$ m filter to remove undissolved particles. 10 mL of filtered *p*-phenylene diamine dihydrochloride/PBS solution is added to 90 mL glycerol and mixed (inversion or vortex). A 3- $\mu$ L aliquot of DAPI counterstain is added to 10 mL anti-fade to give a final concentration of 0.006  $\mu$ g/mL (see **Note 6**).

### 3. Methods

#### 3.1. Conventional Cytogenetics

##### 3.1.1. Culture 1: Overnight Culture

1. Place 10 mL RPMI 1640 medium supplemented with PSG and 10% FCS into a sterile 50-mL tissue-culture flask.
2. Using a sterile pipet, inoculate medium with appropriate amount of bone marrow (see **Note 7**).
3. Lay flask flat and incubate at 37°C for approx 24 h.
4. Add 0.2 mL of 10  $\mu$ g/mL Colcemid to culture and incubate at 37°C for a further 30 min.
5. Transfer culture to harvesting tube and centrifuge for 10 min at approx 200g in a sealed bucket centrifuge.
6. Discard supernatant.
7. Resuspend cell pellet in 8 mL of KCl and place in 37°C water bath for 20 min.
8. Centrifuge for 10 min at 200g.
9. Discard supernatant.
10. Resuspend in 5 mL of fresh fixative (3:1 analar methanol:glacial acetic acid) by adding the fix drop by drop initially with thorough mixing to avoid cell clumping (see **Note 8**).
11. Refrigerate cell suspension at 4°C for 15 min.
12. Repeat **steps 8–10** at least once. The fixative should be replaced until suspension appears clear without any trace of a brown tinge. Finally, the suspension is spun and diluted if necessary with fixative to produce a slightly cloudy appearance (see **Note 9**). The cell suspension should be stored at –20°C until slide making (see **Subheading 3.2.**).

##### 3.1.2. Culture 2: 48-Hour Synchronized Culture

1. Place 10 mL RPMI 1640 medium supplemented with PSG and 10% FCS into a sterile 50-mL tissue-culture flask.
2. Using a sterile pipet, inoculate medium with appropriate amount of bone marrow (see **Note 7**).
3. Lay flask flat and incubate at 37°C for approx 24 h.
4. After incubation for 24 h, add 100  $\mu$ L of combined FdU/uridine solution to the flask and incubate at 37°C overnight (see **Note 10**).
5. The following morning, add 100  $\mu$ L of BrdU (see **Note 11**) and incubate for a further 7 h.
6. To harvest, follow the procedure outline for an overnight culture from **step 4**.

### 3.1.3. Direct Harvest of Peripheral Blood

When a peripheral blood sample is referred solely for interphase FISH analysis, a direct harvest of the buffy coat cells by the following method provides a fixed suspension of interphase cells suitable for interphase FISH.

1. Into a labeled, nonsterile centrifuge tube place 8 mL KCl and warm to 37°C for a minimum of 10 min.
2. Centrifuge peripheral blood tube in a sealed bucket for 10 min at 200g.
3. Take all the buffy coat from all tubes provided and add to warmed KCl.
4. Follow **steps 7 to 12** of the harvest procedure.
5. Store tube in -20°C freezer awaiting FISH analysis.

### 3.2. Slide Making and G-Banding

Slide preparation is important for optimal G-banding. Ideally, slides are made when the temperature is 22°C and the humidity is 40–45% (*see Note 12*).

1. Place cell suspension on the bench and allow it to warm to room temperature. This usually takes approx 15 min (*see Note 13*).
2. Clean slides by filling a Coplin jar with 100% ethanol, dipping slides into ethanol, wiping clean with a lint-free tissue, and allowing to air dry.
3. Using a clean Pasteur pipet, drop three to five drops of suspension evenly along the slide. Allow the slide to air dry.
4. Assess slide quality by phase-contrast microscopy. The chromosomes should appear medium gray in contrast and be well spread (*see Note 14*).
5. The slides should be aged prior to banding to reduce fuzziness and to produce clear, crisp G-bands. There are a number of methods available. The following steps are designed to produce successful G-banding on the day slides are made (*see Note 15*).
6. Allow the freshly made slide to air-dry at room temperature for 30 min (*see Note 16*).
7. Prior to commencing banding, set up one Coplin jar with a working solution of diluted trypsin and two Coplin jars with  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free solution and stand at room temperature.
8. Place slide on hotplate at 100°C for 8 to 10 min (*see Note 17*).
9. Without allowing the slide to cool, dip into the diluted trypsin and agitate for approx 8–10 s (*see Note 18*).
10. Rinse in two changes of  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free solution.
11. Shake off excess moisture and place slide on a staining rack. Pipet Leishman's solution onto the slide and let stand for 8 to 10 min or longer if necessary (*see Note 19*).
12. Rinse stain off under running tap water.
13. Allow slide to air-dry.
14. Cover slip slide by placing three small drops of DPX mounting medium at intervals along the cover slip. Gently place the air-dried slide face down onto the coverslip, invert and place the slide onto a 37°C hotplate for sufficient time to allow the DPX to set.



15. The G-banded slide is now ready for microscopic analysis. All abnormalities are described according to the International System for Human Cytogenetic Nomenclature (ISCN) (1995) (*see Note 20*). An analysis may require more than one slide to be examined per culture, depending on the number and quality of metaphases available. Malignant cells tend to produce metaphase spreads with shorter chromosomes and poorer morphology than their normal counterparts. Abnormal clones may be overlooked if only well-banded metaphases of good morphology are analyzed. The number of metaphases available for analysis on each slide varies greatly. Ideally, the 20 to 40 metaphases required for an adequate analysis will be found on one slide, but it may be necessary to band several slides to obtain sufficient metaphases (*see Note 21*).

### 3.3. Fluorescence In Situ Hybridization

The following method is optimized for Vysis® translocation probes. It includes a co-denaturation step of both probe and target DNA. This method works well for cytogenetic preparations, tissue imprints, and bone marrow smears.

1. Slides should ideally be prepared in advance and aged at least 24 h (*see Note 22*).
2. When making slides, there should be adequate numbers of metaphases or interphase cells for FISH analysis. Place one to three drops of suspension, depending on cellularity, on one area of the slide (*see Note 23*).
3. Locate an interphase/metaphase-rich area of the slide and mark the top of this area with a diamond pencil on the underneath surface of the slide (*see Note 24*).
4. Calculate the volume of probe/hybridization buffer mixture required for the size of cover slip to be used (*see Note 3*).
5. Turn on the dry block heater to 72°C and hotplate to 37°C.
6. Prepare the hybridization box by placing a sponge dampened with 2X SSC in the bottom of the box, heat for 10 s in a microwave, and then place the box into the 37°C incubator.
7. Apply 10 µL premixed probe mixture to each slide using the mark underneath the slide as a guide.
8. Cover with a 24-mm square cover slip and seal the edges with glue/rubber cement immediately after the probe mixture has been applied to avoid drying.
9. Place slides (no more than six at one time) on the 72°C hotplate and denature for 2 min (it is critical that it be no longer than 2 min).
10. Transfer slides to the 37°C hotplate until all slides have been denatured and are ready to place in the hybridization box.
11. Incubate at 37°C. The probe manufacturer recommends that hybridization occur over a 6- to 16-h period; we have obtained successful results using a 3.5-h incubation.
12. Prewarm a Coplin jar filled with 0.4X SSC/0.3% NP40 in the water bath, to the temperature appropriate for each probe (74°C for Vysis translocation probes).
13. Carefully peel off glue from the slide and remove the cover slip.
14. Immediately, immerse the slide in the posthybridization wash solution. Slides (no more than four at a time) should be placed in 0.4X SSC/0.3% NP40 for 2 min.



15. Wash slides in 2X SCC/0.1% NP40 at room temperature for no more than 1 min.
16. Air dry away from light and apply the DAPI/anti-fade counterstain. This should be applied sparingly—one drop is usually sufficient—using a Pasteur pipet.
17. Overlay with a 24 mm × 50 mm cover slip and press down gently with a tissue to spread the DAPI/anti-fade under the cover slip and remove any excess around the edges (*see Note 25*).
18. The slide is now ready for analysis.
19. All FISH studies should be scored by two scientists, preferably within 1 to 2 d of hybridization. For unique sequence probes on diagnostic samples, a minimum of 5–10 metaphases and/or 50 interphase nuclei should be scored. For follow-up cases or suspected mosaicism, a minimum of 200–300 interphase cells should be scored. If signals are too faint or a significant number of cells are unscorable and the control slides are also unscorable, the experiment should be repeated. There are a number of variables that will affect the final FISH result (*see Note 26*). Ultimately, however, a poor result may relate to specimen factors beyond the control of the laboratory.
20. Images should be captured and saved as a record. The VCCS protocol advocates the capture of at least one image for cases with a normal result and at least two images representing clonal abnormalities.
21. FISH reports should include the probe type, the number of cells scored, and a karyotype according to the ISCN (1995) (9) (*see Note 27*).
22. When interphase FISH results are reported, the results should be compared with normal control values. This is particularly important when loss of signals is identified.

#### 4. Notes

1. RPMI 1640 medium, modified with HEPES buffer, is commercially available in liquid form. At the VCCS, medium is made up from powder: 16.4 g RPMI 1640 powder is dissolved in 1 L distilled H<sub>2</sub>O with 2 g sodium bicarbonate, adjusting the pH to 7.2–7.3 using 1 N HCl or 1 N NaOH and sterilizing by filtration through a 0.20-μm filter. It is aseptically decanted into sterile bottles and stored at 4°C for 1 to 2 wk. It should not be used if it becomes opaque, changes color, or has floating particles. This is certainly the most cost-effective method. However, if only small numbers of samples are being cultured, it may be simpler to purchase liquid medium. Once a bottle of medium is opened and ready for use, it will last only a few days at 4°C in the dark. One way to avoid wasting expensive medium is to place 10-mL complete medium aliquots into tissue-culture flasks and freeze at –20°C until required.
2. The G-banding method given here uses Leishman's solution, as we have found this method to be the most reliable and least given to fading over time. However, other methods commonly used in cytogenetics laboratories involve the use of Giemsa stain or Wright's stain.
3. Cover slip size: to ensure adequate coverage of the hybridization area on the slide while avoiding wastage of expensive probe, the minimum possible volume of hybridization mixture plus probe applied to the slide is used with the appropriate-

**Table 1**  
**Probe/Buffer Volume Determines the Cover Slip Size Required**

Recommended cover slip size	Volume of probe/buffer mixture
12 mm round	3 $\mu$ L
18 mm square	6 $\mu$ L
24 mm square	10 $\mu$ L
24 mm $\times$ 50 mm rectangular	20 $\mu$ L

sized cover slip. Too little probe mixture applied under a large cover slip causes air pockets, drying out of the specimen, and areas of no hybridization. Too much probe with a small cover slip causes probe mixture to seep out around the cover slip (*see Table 1*).

4. The glue or rubber cement used must be able to seal the edges of the cover slip to ensure an adequate seal but also be readily removed. We have found bicycle tube glue to be the easiest to use.
5. Premixing of probe with hybridization mixture allows more accurate pipetting of the small volumes of probe required for each experiment and allows the probe to be aliquotted into small volumes to reduce the number of times the probe stock is thawed and re-frozen. It is possible to dilute most probes considerably more than recommended and still achieve good results. The high cost of probes dictates that most laboratories will attempt to use as little as possible. We have successfully used most probes at half the recommended concentration. Using only 3  $\mu$ L probe mixture with a 12-mm round cover slip further reduces the amount of probe used per slide and hence the overall cost of the test. However, all FISH methods should first be established using the recommended concentration of probe.
6. If the DAPI counterstain appears to fluoresce too strongly, such that target signals are being overpowered, it can be diluted further with anti-fade. The final DAPI/anti-fade solution is aliquotted into Eppendorf tubes and stored in the dark at  $-20^{\circ}\text{C}$ .
7. The amount of bone marrow aspirate inoculated into each culture is dependent on the cellularity of the aspirate and the degree of blood dilution. If a cell count is performed on the aspirate, approx  $1 \times 10^7$  nucleated cells should be added to each 10-mL culture. Alternatively, an assessment of the viscosity of the marrow specimen may be made. If the marrow appears to be quite thick and viscous, add 5 to 7 drops to each culture using a sterile pipet; if only slightly viscous, add 8 to 11 drops; and if quite thin and blood diluted, add 12 to 14 drops of marrow. Bone marrow from a new case of CML may readily overgrow; if the marrow specimen is thick and sticky, use only one to three drops; if of medium viscosity, three to four drops; and if thin or very thin, five to eight drops of marrow. In general, care should be taken not to exceed approx 12 to 14 drops of marrow, as an excess of red cells added to the culture may affect cell growth. If bone marrow is not avail-

able, peripheral blood may be used, provided there are sufficient cells capable of spontaneous division in the peripheral blood. Peripheral blood samples should be inoculated as for thin marrow, taking into account the white blood cell count (WBC) (i.e., fewer drops when high WBC). Note, however, that greater than eight drops tends to result in excessive red cell contamination and a poor suspension after fixing. When the WBC is not given, the specimen should be spun at 200g for 10 min and the size of buffy coat observed. If there is a small buffy coat layer, indicating a low WBC, set up from the buffy coat interface. If the buffy coat layer is large, remix the sample and use whole blood. In the case of a newly diagnosed CML specimen, when only peripheral blood is available, whole blood should always be used rather than buffy coat. Occasionally, bone marrow trephine samples may be induced to yield analyzable metaphases, but the success rate with trephines is generally low. Trephine specimens should be scraped or chopped up under sterile conditions, to produce a single-cell suspension that can then be used for inoculation.

8. All harvesting to the point of first adding fixative, should be performed wearing disposable gloves and in a class II biohazard cabinet. All centrifuging to the first fix stage should be in a centrifuge with sealed, autoclavable buckets.
9. An overcrowded suspension indicates that the culture was over-inoculated. If a culture yields more than 8 mL of cloudy cytogenetic suspension, a repeat culture should be attempted using less marrow, if possible. Overcrowding may result in few metaphases of poor morphology.
10. The blocking period should not be less than 14 h or greater than 17 h (10).
11. The incorporation of BrdU into the DNA renders the chromosomes susceptible to degradation on exposure to ultraviolet light; therefore, the culture should be shielded from light. Because modern incubators are light-proof, no special precautions are required. However, with an incubator that allows light entry when the door is closed, the flasks should be placed in a light-proof box inside the incubator after the addition of BrdU.
12. High humidity causes slides to dry too slowly and thus chromosomes to over-spread. If humidity is above 45–50%, the slide may be warmed briefly on a hotplate prior to dropping suspension onto the slide. Alternatively, low humidity causes slides to dry too fast, and so chromosomes become clumped and under-spread. Below 40% humidity, slides may be rested on a freezer block for a few seconds prior to dropping suspension onto the slide. An alternate method of slide making when the humidity is low is to use cell suspension that has just been removed from the freezer, rather than allowing the suspension to warm to room temperature.
13. Small amounts of suspension should not be left uncapped on the bench at room temperature for long periods of time. As soon as slides have been made, recap and store suspension in the freezer.
14. The following steps may be tried to enhance spreading: (1) breathe warm, moist air onto a clean slide and drop sample onto the misted surface; (2) place clean slides in freezer until surface is misted, then drop as described previously; (3)

hold slide on an angle when dropping suspension; (4) dip slide in a 60% acetic acid solution and drop sample onto the wet slide. To reduce overspreading, the slide may be heated on a 60°C hotplate prior to dropping suspension onto slide. Traditionally, cytogeneticists claimed that the height from which the cell suspension was dropped onto the slide was critical for successful spreading. However, in truth, there is no advantage to dropping suspension from a great height and considerable disadvantage if the best metaphases end up on the floor.

15. Slides may be “aged” by a variety of methods, including placing slides in a 60°C oven or in a desiccator for 1–2 d or as long as required.
16. Slides can continue to be aged in this manner for a number of hours after being made (30 min is not a critical time period). However, it works well only if performed the same day on which slides are made.
17. While most slides require between 8 and 10 min on the 100°C hotplate to age sufficiently, up to 20 min may be required in some cases when banding is being attempted on the same day as slides are prepared.
18. The time needed for each slide to be immersed in trypsin may vary depending on the quality of the chromosome morphology; less time may be required for chromosome preparations of poor quality. One slide should be tested at a time to estimate the optimum time for producing satisfactory G-bands. If the banding is not distinct, the time in trypsin may be extended. If the chromosomes appear fuzzy, the slides may need to be aged longer, either by increasing the time on the 100°C hotplate or by leaving overnight in a 60°C oven.
19. Hotplate-aged slides tend to be paler staining than slides aged by alternative methods, and so may require a longer application of Leishman’s solution.
20. Only clonal abnormalities can be included in the karyotype. Thus, structural abnormalities or gains of whole chromosomes must be observed in at least two metaphases, and loss of a whole chromosome must be observed in at least three metaphases to establish the clonality of an abnormality.
21. A failed culture may result from a number of factors. The cause may be sample related, with few cells capable of division due to relative aplasia or blood dilution of the sample, prior therapy with chemotherapy agents, too long in transit, inappropriate storage in transit (either too hot or too cold conditions), inappropriate anticoagulation (ethylenediamine tetraacetic acid [EDTA]), too much anticoagulant (hemolyzing the sample) or too little (allowing the marrow to clot). Of the laboratory factors that may contribute to culture failure, the medium is critical; it must be maintained at the correct pH, and a careful check of the color of the medium provides an indication of whether the pH has changed.
22. For an urgent FISH study using a freshly made slide, pre-treatment with 2X SSC will obviate the need for aging the slide. The slide is immersed in 2X SSC in a Coplin jar at 37°C for 30 min. The slide is then dehydrated through an ethanol series—placed for 1 min each in 70%, 80%, and finally 100% ethanol and then air dried.
23. To ensure that adequate numbers of metaphases and interphase cells are available for FISH analysis, there should be 20 or more interphase cells per low-power field and 30 or more metaphases in the area of a small cover slip (12 mm<sup>2</sup>) if

- metaphase FISH analysis is required. Optimally, at least 80 interphase cells and 2 or more metaphases per high-power field should be observed.
24. Slides should be marked on only one side of the hybridization area, as multiple marks with a diamond pencil render the slide more fragile and prone to breaking.
  25. Any mixing of DAPI/anti-fade on the surface of the cover slip with oil used to visualize the slide through an oil-immersion lens creates an opaque smear, through which microscopic analysis is impossible.
  26. FISH troubleshooting: if FISH results obtained are less than optimal, there are a number of areas that can be altered to improve results. Cross-hybridization may be improved by increasing the posthybridization wash temperature or decreasing co-denaturation time or temperature. High levels of background on the slide may be due to the slides not being cleaned sufficiently prior to dropping suspension, or inadequate posthybridization washes. Ensure that wash solutions are made up correctly and at the correct temperature, remove cover slip, and repeat wash. Weak or no signal may be caused by many factors. The slide and/or probe may be inadequately denatured; increase denaturation temperature or denaturation time by 2 to 4 min. The premixed probe may have been incorrectly diluted or not well mixed prior to use. The wash conditions may be too stringent, in which case, decrease hybridization temperature or increase salt concentration in posthybridization wash solutions to 0.2X SSC. Probe or specimen may have been stored improperly—probes must be stored at  $-20^{\circ}\text{C}$ , protected from light; and if slides are made more than a few days in advance of FISH being performed, they should be stored at  $-20^{\circ}\text{C}$ . Use of the incorrect filter set on the fluorescence microscope may lead to the inference that the experiment has been unsuccessful. The filter set on the fluorescence microscope must be appropriate for the probe fluorophore being used; multi-bandpass filter sets provide less light than single-bandpass filters, and so probes may appear fainter when viewed through a multi-bandpass filter.
  27. FISH nomenclature was included in the 1995 version of the ISCN (9). However, its use is problematic, with extraordinary variation observed in its application (11).

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