LEAD ARTICLE

# Analysis of Genotype and Phenotype on the Same Interphase or Mitotic Cell

## A Manual of MAC (Morphology Antibody Chromosomes) Methodology

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**ABSTRACT:** *The purpose of this paper is to serve as a MAC (Morphology Antibody Chromosome) manual describing combined methodologies that allow simultaneous and/or sequential analysis of cell morphology, immunophenotype, and banded chromosomes and/or in situ hybridization signals. The MAC techniques used at the Department of Medical Genetics of the University of Helsinki, Finland, are described and modifications or related techniques reported by other authors are discussed. A list of references concerning applications is also given.*

## INTRODUCTION

The MAC (Morphology Antibody Chromosomes) method was first described in 1984 for use in mitotic cells [1]. With the advent of molecular cytogenetics, the MAC was applied to interphase cells [2]. At present, the MAC and its modifications allow simultaneous and sequential analysis of phenotype (cell morphology and immunophenotype) and genotype (karyotype or in situ hybridization signals) on a single mitotic and/or interphase cell [3, 4]. The main applications have been in the study of lineage involvement of chromosomal abnormalities in neoplastic and normal tissue, and in the fields of cell proliferation kinetics and clastogenesis (Table 1, reviews by Knuutila and Teerenhovi [3], Köller et al. [5], and Larramendy et al. [6]). Accompanying the development of molecular cytogenetics and other cell biologic techniques, new modifications of the MAC have been recently adopted [7–13]. This paper reviews the present techniques for simultaneous and sequential analysis of phenotype and genotype, and serves as an updated manual of the MAC.

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## METHODOLOGY

### General Outline

Irrespective of whether metaphase or interphase cells are studied and whether chromosome banding or in situ hybridization techniques are employed in the genotypic study, MAC is the abbreviation used for the combined methodology. Figure 1 is a schematic presentation of MAC procedures. In the MAC, immunophenotype and in situ hybridization signals can be studied simultaneously (simultaneous analysis) or sequentially (sequential analysis).

### Preparations

The MAC is applicable to cytospin preparations of cell suspensions as well as tissue sections, smears, and cells cultured in situ. If the MAC study is performed on metaphase cells, a Colcemid (0.4 μg/ml, Difco Laboratories, Detroit, MI) treatment of up to 24 h is recommended in order to obtain the maximum number of metaphases. Before culturing, erythrocytes present in the sample are removed by dextran sedimentation [14] or density gradient centrifugation using Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden), or Percoll (Pharmacia Fine Chemicals), following the procedures of Julius et al. [15] and Timonen and Saksela [16]. Mononuclear leukocytes isolated by the aforementioned procedures can be further treated using immunomagnetic beads (Dynabeads M-450, Dynal A.S., Oslo, Norway) coated with appropriate monoclonal antibodies [17], to separate different cell subsets.

### Hypotonic Treatment of Cell Suspensions

*Procedure:* The cells are suspended for 5 minutes in a solution containing several physiologic ions, glycerol, and sucrose at room temperature. The working solution comprises



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**Table 1** List of references for application of MAC and related techniques

| | |
|---|---|
| Lineage involvement of chromosomal abnormalities | |
| Normal bone marrow/ploidy | Larramendy et al. [70] |
| Normal kidney/polysomy 7 | Dal Cin et al. [71] |
| Chronic myeloid leukemia | Rastrick [72] |
| | Bernheim et al. [73] |
| | Teerenhovi et al. [1] |
| | Knuutila and Teerenhovi [3] |
| | Knuutila et al. [4, 74] |
| Myelodysplastic syndrome | Parlier et al. [75] |
| | Kibbelaar et al. [8] |
| | Nylund et al. [76] |
| Polycythemia vera | Price et al. [12] |
| | Kanfer et al. [100] |
| Acute myeloid leukemia | Keinänen et al. [42] |
| | Kere et al. [33] |
| | Stamberg et al. [77] |
| | Knuutila et al. [74] |
| Chronic lymphocytic leukemia | Knuutila et al. [78] |
| | Autio et al. [79] |
| | Larramendy et al. [80] |
| | Pérez Losada et al. [81] |
| Acute lymphoid leukemia | Anastasi et al. [82] |
| | Knuutila et al. [69] |
| Non-Hodgkin lymphoma | Franssila et al. [83] |
| | Lindholm et al. 1989 [84] |
| | Knuutila et al. [85] |
| Hodgkin lymphoma | Teerenhovi et al. [86] |
| Angioimmunoblastic lymphadenopathy | Schlegelberger et al. [43, 87] |
| Glioblastoma | Knuutila et al. [4] |
| Endometrial polyp | Fletcher et al. [46] |
| Pulmonary chondroid hamartoma | Fletcher et al. [45, 47] |
| Breast adenofibromas | Fletcher et al. [45] |
| Cell proliferation | |
| Bone marrow cells | Keinänen et al. [35, 42, 88, 89] |
| Lymphocytes | Knuutila and Kovanen [90] |
| | Kovanen and Knuutila [91] |
| | Kovanen et al. [92–94] |
| | Larramendy and Knuutila [49] |
| | Larramendy et al. [6] |
| Micronucleus test | |
| Peplomycin | Slavutsky et al. [95] |
| Smoking | Larramendy and Knuutila [96] |
| Review article | Knuutila and Teerenhovi [3] |
| | Knuutila [30] |
| | Köller et al. [5] |

one part of hypotonic solution [50.0 mM glycerol, 5.0 mM KCl, 10.0 mM NaCl, 0.8 mM $MgCl_2$, 1.0 mM $CaCl_2$, and 10.0 mM sucrose (pH 7.0)] and one part of RPMI 1640 culture medium (GIBCO, Grand Island, NY) containing 20% fetal calf serum [1].

*Comments:* A mild and cell membrane-stabilizing hypotonic solution is used for metaphase cells of all kinds of preparations to obtain sufficient chromosome spreading inside an intact cell membrane. Still, hypotonic treatment may dramatically change the cell morphology. If the MAC is to be applied to interphase cells only, hypotonic treatment should be avoided.

The hypotonic solution usually employed is a modification of the medium proposed by Stenman et al. [18].

Perry and Thomson [19] used a simplified hypotonic treatment, with a mixture of 2 ml of culture medium and 2 ml of hypotonic solution consisting of culture medium diluted with distilled water (1:4) and 0.4% glycerol (10 minutes at room temperature).

Differences in the spreading of chromosomes in the metaphase plate are frequently observed. For example, metaphases belonging to the erythrocytic cell lineage are sometimes found to be poorly spread. Better spreading can then be achieved by increasing the concentration of the hypotonic solution in the working solution; elevated G values dur-

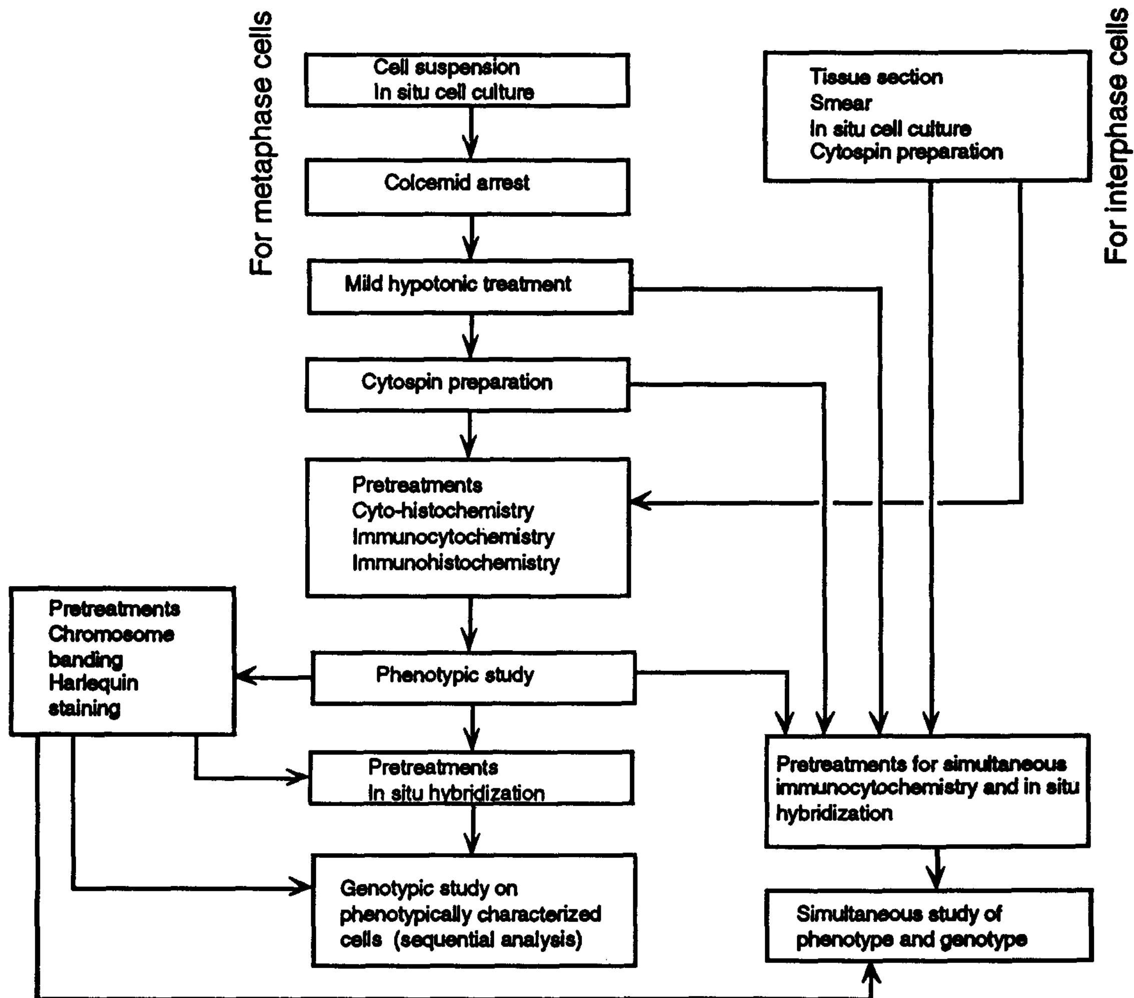


**Figure 1** Main steps in the MAC (Morphology Antibody Chromosomes) methodology for interphase and mitotic cells showing the combination with chromosome banding, sister-chromatid differentiation, and in situ hybridization procedures.

ing cytocentrifugation (see Cytocentrifuge Slide Preparations, *Procedure*) are also conducive to improved spreading. Cells such as Reed-Sternberg cells in Hodgkin's disease or megakaryocytes in bone marrow samples are very sensitive to hypotonic treatment, which tends to destroy them or disrupt their cell membranes. To avoid this, the hypotonic treatment should be very mild or totally disregarded. Best results with Reed-Sternberg cells are obtained when the culture medium is used as the "hypotonic solution," and with megakaryocytes when the concentration of the culture medium in the hypotonic solution is increased.

### Cytocentrifuge Slide Preparations

*Procedure:* After hypotonic treatment, the cell suspension is pipetted into cytocentrifuge chambers (80–150 μl each, $4 \times 10^4$–$7.5 \times 10^4$ cells/chamber) and centrifuged at 400 g for 3–5 minutes onto ethanol-cleaned slides (Cytospin, Shandon Elliot, Runcorn, U.K.). The slides are allowed to dry at room temperature overnight [1].

*Comments:* Cellular immunophenotype is well identifiable on slides kept at room temperature up to 3 weeks. Alternatively, the slides can be stored as such at −20°C or −70°C, or in absolute ethanol at 4°C or at −20°C. Prior to immunocytochemical procedures and in situ hybridization, the slides are allowed to dry at room temperature for 12–24 hours.

Haas et al. [20] and Köller et al. [5] reported that good spreading of metaphase chromosomes and improved adhesion of cells can be obtained by using poly-L-lysine-coated slides [21] for cytocentrifugation.

### Hypotonic Treatment of In Situ Cell Cultures

*Procedure:* Colcemid-treated cells growing in a tight monolayer on a surface in a culture flask (e.g., fibroblasts, meso-

thelioma, neuroblastoma, osteosarcoma, and other tumor cells) can be detached from the growth surface by trypsinization to make a cell suspension. The suspension is then processed as described above. However, it is also possible to apply hypotonic solution to a monolayer of cells directly in a tissue culture chamber (Flaskette Chamber Slide; Nunc, Naperville, IL) after mitotic arrest. After air-drying for 12–24 hours, the cells are ready for cytochemical or immunocytochemical characterization [3, 22].

*Comments:* Treatment of in situ cell cultures has the advantage over cytogenetic preparations made after trypsinization of cell monolayers that often the number of mitoses is higher, as no dividing cells are lost during the enzymatic digestion or during rinsing.

Henn et al. [23] used 0.0375 M KCl (8–14 minutes, room temperature) for hypotony, followed by fixation with methanol (−20°C, 5 minutes), and acetone (1 minute, room temperature).

**Paraffin-embedded Tissue Sections**

*Procedure:* Normal or tumor tissue samples are immersed in 7.5% formaldehyde in phosphate buffer for several hours until embedded in paraffin. Thin sections (6 µm) are cut and fastened on slides coated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO) [21] or Evo Stick wood adhesive [25], and then incubated at 60°C overnight.

The tissue sections are deparaffinized in xylene (3 times, 10 minutes each) and in an alcohol series (99%, 94%, 70%, 5 minutes each) and finally air-dried. Blocking of endogenous peroxidase activity, when required, is achieved by treating the slides in methanol containing 1% $H_2O_2$ at room temperature for 30 minutes. After a wash in methanol and air-drying for 12–24 hours, the slides are ready for cytochemical or immunocytochemical staining.

**Frozen Tissue Sections**

*Procedure:* Tissue material frozen in liquid nitrogen is cut by cryostat into 5–6-µm sections at −20°C. The sections are placed on aminoalkylsilane-treated glass slides and allowed to dry for 24 hours, after which the slides are ready for cytochemical or immunocytochemical staining [10, 24–28].

**Smear**

*Procedure:* The technique of preparing cell smears can be found in many textbooks and atlases of hematology, e.g., Williams [29].

## CYTOCHEMISTRY/IMMUNOCYTOCHEMISTRY

In principle, all cytochemical and/or immuno-cytochemical procedures, such as Giemsa, May-Grünwald-Giemsa, Sudan Black B (SBB), α-naphthyl acetate esterase (ANAE), immunofluorescence, immunoperoxidase (IP), and alkaline phosphatase anti-alkaline phosphatase (APAAP), can be applied to MAC. The first step involves fixation, the nature of which is determined by the techniques of cell classification and chromosome staining to be used.

**Giemsa Staining for Routine Cytologic or Cytogenetic Analysis**

*Procedure:* The slides are fixed in a mixture of methanol and formaldehyde (50.0 ml methanol, 25–250 µl 37% formaldehyde) for 5–10 minutes, washed in methanol, and air-dried at room temperature for 2–5 minutes. The slides are then stained with 5% Giemsa (Merck Diagnostica, Darmstadt, Germany) in Sörensen buffer (pH 6.8) for 5–15 minutes, rinsed with running tap water, and air-dried.

**May-Grünwald-Giemsa Staining**

*Procedure:* The slides are fixed in a mixture of methanol and formaldehyde (50.0 ml methanol, 25 µl 37% formaldehyde) for 15 minutes and air-dried at room temperature for 2 minutes. The slides are then stained with May-Grünwald-Giemsa (Merck) for 20 minutes, washed three times (2 minutes each) with phosphate buffer at pH 6.8 (Buffer Tablets pH 6.8, BDH Limited, Poole, U.K.), counterstained with 5% Giemsa solution in Sörensen buffer (pH 6.8) for 10 minutes, rinsed with running tap water, and air-dried.

*Comments:* May-Grünwald-Giemsa staining is used in studying the basic morphology of bone marrow cells. Neutrophilic, eosinophilic, and basophilic granulocytes, lymphocytes, and monocytes can be distinguished on the basis of the dark-blue to violet color of the nuclei, the different shades of pale blue of the cytoplasms, the red of the eosinophil granules, the dark-blue of the basophil granules, and the purplish shade of the neutrophil granules.

**SBB Staining**

*Procedure:* The slides are fixed in 37% formaldehyde for 1 minute and washed under running tap water for 2 minutes. The cells are then stained with SBB (Merck) for 30 minutes, washed with 70% ethanol for 2 minutes, and washed under running tap water for 2 minutes. Finally, the slides are counterstained with 5% Giemsa solution in Sörensen buffer (pH 6.8) for 10 minutes or with fluorescent quinacrine mustard (0.5% in distilled water, Sigma) [4, 30].

*Comments:* SBB staining recognizes lipofuscins and ceroids [31]. SBB-positive cells, e.g. granulocytes, have black granules and a pale gray cytoplasm [31].

**ANAE Staining**

*Procedure:* The slides are fixed in formol calcium fixative, prepared by mixing 100 ml of 37% formaldehyde, 100 ml of 0.9 M $CaCl_2$, and 900 ml of distilled water (pH 6.7, 4°C, 10 minutes). Then the slides are washed in distilled water for 20 minutes and stained by incubating them in a mixture of 25 ml of α-naphthyl acetate (Sigma) in acetone (10 mg/ml), 72.5 ml of 0.2 M $NaH_2PO_4 \cdot H_2O$, 25 ml of distilled water, 4 ml of 0.6 M $NaNO_2$, and 4 ml of pararosaniline hydrochloride in 2 N HCl (0.4 g/ml) at 37°C for 10–20 minutes. Finally, the slides are washed in running tap water and counterstained with 5% Giemsa solution in Sörensen buffer (pH 6.8) for 15 minutes, rinsed with running tap water, and air-dried [32, 33].

*Comments:* The substrates used for the demonstration of esterases, and even lipases and cholinesterases, can be hydro-

lyzed by a number of enzymes. The method used by us employs α-napthyl acetate as the substrate, leading to the release of α-naphthol during the hydrolysis. The α-naphthol is then coupled with a suitable diazonium salt, such as the pararosaniline in the technique proposed by Davis and Ornstein [32], to produce an insoluble azo dye at the site of enzyme activity. ANAE staining is used to identify monocytes.

To obtain high-quality ANAE staining, it is important that equal parts of $NaNO_2$ and pararosaniline hydrochloride solutions are mixed together before adding them to the incubation medium and that the pH is adjusted to 7.4, if necessary, with 0.2 M $NaH_2PO_4 \cdot H_2O$.

**Indirect Immunofluorescence Staining**

*Procedure:* The slides are fixed in a mixture of acetone and formaldehyde (50 ml acetone, 40 µl 37% formaldehyde) at room temperature for 1 minute and washed briefly in 50 ml phosphate-buffered saline containing 40 µl fetal calf serum (PBS-FCS). After fixation and washing, the mouse anti-human primary monoclonal antibody (1:10–30 in PBS-FCS) is applied directly to the wet slides, which are then incubated in a moist chamber for 30 minutes and finally washed twice with PBS-FCS. Tetramethylrhodamine (TRITC)-conjugated goat anti-mouse antibody (1:200, Cappel, Cochranville, PA) is then applied to the slides, and the slides are incubated in a moist chamber for 30 minutes, then washed with PBS-FCS. Finally, the cells are counterstained with quinacrine mustard (0.5% in distilled water, Sigma). TRITC-positive cells and Q-banded chromosomes can be examined under a fluorescence microscope equipped with appropriate filter combinations [1, 34].

*Comments:* Formaldehyde fixation can be only rarely applied to fluorescent monoclonal antiserum staining. Commercially available stocks of formaldehyde often contain impurities, both organic and inorganic (e.g., formic acid, methanol), which may interfere with histochemical and/or immunohistochemical reactions. As a substitute, paraformaldehyde (methanol-free formaldehyde) fixation can be used. For our purposes, however, formaldehyde is the better fixative of the two since paraformaldehyde renders the chromosomes fuzzy after Giemsa or quinacrine staining.

Weber-Matthiesen et al. [9, 11] have adopted fixation in acetone at room temperature for 10 minutes. For visualization of the immunophenotype, they use mouse monoclonal antibodies, aminomethylcoumarin acetic acid (AMCA)- or Texas Red-conjugated goat anti-mouse antibodies and rabbit anti-goat antibodies.

**SBB and Immunofluorescence Double Staining**

*Procedure:* The slides are fixed in 37% formaldehyde at room temperature for 1 minute and washed under running tap water for 2 minutes. The slides are stained with SBB (for details of the technique, see SBB Staining, *Procedure*) for 30 minutes, washed with 70% ethanol for 2 minutes, and air-dried for 10 minutes. The slides are then refixed in methanol-formaldehyde (50 ml methanol, 250 µl 37% formaldehyde) for 1 minute, and stained by the immunofluorescence method. Finally, the slides are stained with quinacrine mustard (0.5% in distilled water, Sigma) or 5% Giemsa solution in Sörensen buffer (pH 6.8) for 10 minutes, rinsed in running tap water, and air-dried [30, 35].

*Comments:* With a Leitz fluorescence microscope, the N2 filter is used for TRITC and the E2 for quinacrine mustard. TRITC-positive cells and Q-banded chromosomes can be visualized simultaneously with an Omega double band filter (Omega Dualband, Brattleboro, VT). On the other hand, positive interphases and mitoses stained with SBB are imaged without fluorescence filter under visible light.

**IP Staining**

*Procedure:* The slides are fixed in a mixture of acetone and formaldehyde (50 ml acetone, 40 µl 37% formaldehyde) at room temperature for 1 minute, washed briefly in PBS containing 5% fetal calf serum (PBS-FCS), and incubated with normal horse serum (1:50 in PBS-FCS, Vectastain ABC kit, vector, Burlingame, CA) in a moist chamber for 30 minutes. The preparations are then incubated in a moist chamber successively with mouse anti-human primary monoclonal antibody (1:10–30, 60 minutes), biotinylated secondary antibody (1:250, 30 minutes), and finally with avidin DH-biotinylated horseradish peroxidase H complex (1:160, 30 minutes). Between each step, the slides are briefly rinsed with PBS-FCS. A 3-amino-9-ethylcarbazole substrate is prepared by dissolving 10 mg of the compound (Sigma) in 3 ml N, N-dimethylformamide (Sigma) and mixing this solution with 50 ml sodium acetate-acetic acid buffer (74 ml of 0.2 M acetic acid, 176 ml 0.2 M $CH_3COONa \cdot 3H_2O$, and 250 ml distilled water; pH is adjusted to 5.0 and distilled water is added to make 1 L). Just before staining, 50 µl of $H_2O_2$ (Sigma) is added. The slides are incubated in the presence of the 3-amino-9-ethylcarbazole substrate for 20 minutes. After staining, the slides are briefly washed with PBS-FCS and distilled water, and air-dried at room temperature overnight. The slides are then counterstained with 5% Giemsa in Sörensen buffer (pH 6.8) for 15 minutes, rinsed with running tap water, and air-dried [36–38].

*Comments:* The above procedure is a modification of the technique originally proposed by Kaplow [39]. The IP method is unsuitable for bone marrow cells with intrinsic peroxidase activity unless the peroxidase activity is inhibited by incubating the slides in $H_2O_2$ after fixation. As such blocking may affect the cell morphology in bone marrow samples, other immunostaining methods, such as APAAP staining, are recommended for MAC studies.

Haas et al. [20] used the procedure described by Köller et al. [40], employing glucose-oxidase and glucose during the IP staining, especially when studying myeloid cells. Mixtures of these two reagents continuously generate small amounts of $H_2O_2$, which efficiently inhibits endogenous peroxidase activity.

**APAAP**

*Procedure:* The slides are fixed in cold (4°C) formalin-acetone buffer (0.11 M $Na_2HPO_4 \cdot 2H_2O$, 0.07 M $KH_2PO_4$, 30 ml distilled water, 45 ml acetone, 2.5 ml–25 ml 37% formaldehyde) for 1 minute and then washed in running tap water for 1 minute and with tris(hydroxymethyl)aminomethane-

(TRIS)-buffered saline (pH 7.6) containing 2.5% human AB serum for 10 minutes. The slides are then placed in a moist chamber and treated successively with a mouse anti-human primary monoclonal antibody (1:20–1:50, 30 minutes), rabbit anti-mouse immunoglobulin antiserum (1:25, 30 minutes, Dakopatts, Glostrup, Denmark), and APAAP mouse immunocomplex (1:25, 30 minutes, Dakopatts). Between each immunologic step, the slides are washed in TRIS-buffered saline containing 2.5% human AB serum for 10 minutes. Finally, the slides are treated with APAAP substrate, consisting of naphthol AS-MX phosphate (0.2 mg/ml in dimethylformamide, Sigma) and levamisole hydrochloride (to block endogenous alkaline phosphatase activity; 1 mM, Sigma), by filtering the substrate onto the slides through a 45-µm filter membrane (Millipore, Bedford, MA). The slides are incubated with the substrate for 20 minutes, washed in running tap water for 2 minutes, and air-dried at room temperature overnight. The cells are then counterstained with 5% Giemsa solution in Sörensen buffer (pH 6.8) for 15 minutes [4, 41, 42].

*Comments:* The APAAP reaction can be intensified by repeated incubation with rabbit antimouse immunoglobulin antiserum (1:25, 30 minutes, Dakopatts) and APAAP mouse immunocomplex (1:25, 30 minutes, Dakopatts) before applying the substrate. Similar results are obtained if heat-inactivated FCS (55°C, 30 minutes, GIBCO) is used instead of human AB serum.

The reasons for using formaldehyde instead of paraformaldehyde for fixation have been addressed above (see Immunofluorescence Staining, *Procedure*). The lower formaldehyde concentration yields a stronger positive reaction on APAAP staining, especially when weak antibodies are used, and may enhance the hybridization efficiency in subsequent in situ hybridization procedures. It should be noted that, compared with the former fixative mixture, the decrease in formaldehyde concentration renders the morphology of both chromosomes and cells fuzzier after Giemsa staining. This is particularly evident with chromosome banding.

Besides the fixation procedure discussed above, the one proposed by Schlegelberger et al. [43] has also been found good in our laboratory. This latter comprises fixation in chloroform for 30 minutes, and then in acetone for 10 minutes (both at room temperature). Positive immunophenotype by APAAP and subsequent R-banding are also achieved with this procedure.

### Chromosome Banding

*Procedure:* Mitotic cells are first identified either by cytochemical staining with Giemsa (for details of the technique see Giemsa Staining, Procedure) or by immunologic methods, such as immunofluorescence, IP, or APAAP (for details of the technique, see Procedure under Immunofluorescence, IP, and APAAP Staining). Following cell identification, the slides are destained with 94% ethanol (10 minutes, room temperature), refixed in methanol:acetic acid (3:1) at room temperature for at least 1 hour, and then air-dried at 60°C for 18–24 hours until band induction.

For G-band induction, the slides are bathed in Sörensen buffer (pH 6.8) at room temperature for 17 hours and then stained with 2% Giemsa solution in Sörensen buffer (pH 6.8) for 3 minutes. Then the slides are rinsed with running tap water and air-dried at room temperature. G-banding can also be achieved by digesting the slides with trypsin (0.025%, GIBCO) for 15 seconds up to 60 minutes before washing in distilled water and Giemsa staining.

C-bands are induced by incubating the slides in 0.2 N HCl at room temperature for 1 hour, followed by treatment with saturated $Ba(OH)_2$ solution at room temperature for 10 minutes and Sörensen buffer (pH 6.8) at room temperature overnight. The slides are then stained with 2% Giemsa in Sörensen buffer (pH 6.8) for 3 minutes. Finally, the slides are rinsed with running tap water and air-dried at room temperature.

Simultaneous chromosome banding and determination of immunophenotype can also be achieved when fluorescence systems are used to identify the phenotype of cells in which the chromosomes have been counterstained with quinacrine mustard or Hoechst 33258 (for details of the technique, see Immunofluorescence Staining, *Procedure*) [30, 34, 97–99].

*Comments:* Pérez et al. [44] achieved G-banding in immunophenotyped metaphases after removal of the stain with methanol:acetic acid (3:1, 1–2 minutes) and air-drying of the slides at 50°C. The slides were then incubated in 2 × SSC (standard saline citrate) at 65°C for 10–30 seconds and stained with Wright stain (0.25% in methanol) diluted 1:3 in Sörensen buffer pH 6.8 for 5–10 minutes.

Fletcher et al. [45–47] reported a novel approach to the analysis of APAAP positivity in conventionally harvested cells with Q-banded chromosomes. Despite the use of 0.075 M KCl for hypotony and acetic acid-methanol for fixation, immunostaining with APAAP works properly. Following APAAP staining, the slides are air-dried, counterstained with 0.005% quinacrine mustard dihydrochloride (Sigma) for 10 minutes, rinsed in distilled water for 2 minutes, and air-dried. Under fluorescent light, quinacrine banding and APAAP staining are assessed simultaneously using a BP436/8 + BP excitation Carl Zeiss filter.

Recently, Schlegelberger et al. [43] described modifications of the procedure for simultaneous analysis of the immunophenotype and Q-banding pattern of chromosomes as well as for sequential analysis of the APAAP-positive phenotype and fluorescent R-banding. Immunophenotype is demonstrated by a three-step immunofluorescence technique. After immunostaining, the cells are refixed in methanol:acetic acid (3:1) for at least 1 hour and air-dried. Afterwards, Q-bands are induced by staining the slides in 0.05% quinacrine mustard for 90 seconds, washing with 95% ethanol and then with distilled water, and mounting in McIlvaine's citric acid-$Na_2HPO_4$ buffer at pH 7.4. Immunophenotype and chromosome banding pattern are analyzed simultaneously by using appropriate filter combinations in a fluorescence microscope. For sequential analysis of immunophenotype and R-banding, the mitoses are classified by APAAP and hemalum chromosome counterstaining. After recording of the immunophenotype, the slides are washed in TRIS buffer at 37°C to remove the cover slips and then refixed in methanol-acetic:acid (3:1) for at least 24 hours. Finally, R-banding is

achieved using chromomycin A3 (Sigma) and methyl green (Sigma). Briefly, the slides are stained with 0.05% chromomycin A3 (Sigma) in McIlvain's citric acid-$Na_2HPO_4$ buffer, pH 6.8, containing 5 mM $MgCL_2$ for 20 minutes and then counterstained with 0.5 mM methyl green, pH 6.0 (Sigma) for 3–5 minutes.

**Sister Chromatid Differentiation**

*Procedure:* Cells to be studied are grown in the presence of the base analogue 5-bromo-2-deoxyuridine (BrdU, 33 μM, Sigma) at least for a period equivalent to two cell cycles. During the last 3–4 hours of culture, the cells are treated with Colcemid (0.4 μg/ml, Difco Laboratories). The cells are then processed into MAC slide preparations and their immunophenotype is determined according to the IP method (for details of the technique see IP Staining, *Procedure*). Metaphases positive for each monoclonal antibody are localized and photographed. The slides are then processed as follows.

The IP stain is removed with methanol:acetic acid (3:1, 1–4 hours, room temperature) and the slides are air-dried for 20–24 hours. The slides are then incubated in Hoechst 33258 solution (1 μg/ml in 0.1 M phosphate buffer, pH 6.8, Sigma) for 20–30 minutes, air-dried, mounted in 0.1 M phosphate buffer (pH 6.8), and exposed to UV light delivered by a 15-W UV tube (Philips, The Netherlands) for 1–2 hours at a distance of 10 cm. After the UV exposure, the slides are rinsed in distilled water, air-dried, and processed according to the procedure of Korenberg and Freedlander [48]. In the latter procedure, the slides are incubated in 1 M $Na_2HPO_4$ (pH 8.2–8.4, 5–10 minutes, 88°C), rinsed three times with distilled water, and stained with 5% Giemsa solution in Sörensen buffer. Finally, the slides are rinsed with running tap water and air-dried at room temperature [6, 49].

*Comments:* The method described above is a combination of the MAC procedure and the fluorescence-plus-Giemsa methods for sister chromatid differentiation proposed by Perry and Wolff [50] and Korenberg and Freedlander [48], with some modifications. By re-examining the previously immunologically characterized metaphases, it is possible to determine both the number of cell cycles these cells have undergone after the introduction of BUdR into the culture medium and the proportion of first, second, third, or subsequent mitoses in the different cell subsets.

Recently we published an immunologic modification of the sister chromatid differentiation procedure described above [51]. At present we are adapting this staining to MAC methodology for simultaneous study of the immunophenotype and the sister chromatid differentiation pattern.

**Pretreatment for In Situ Hybridization**

Pretreatment of immunophenotyped cells for non-radioactive in situ hybridization (NISH) varies depending on whether sequential or simultaneous analysis is performed, as well as on the type of preparation studied.

*Procedure for Sequential Analysis:* Previously immunostained cytospin or in situ culture preparations are refixed and destained with methanol-acetic acid (3:1) for 1 hour. Cytoplasm is then removed by pepsin treatment (0.01–0.1 mg/ml in 0.01 N HCl, Sigma) in 37°C for 4–10 minutes, followed by washing in distilled water or PBS and dehydration in an ethanol series. To improve the morphology of the cells, the slides may be postfixed in 1% formaldehyde in PBS containing 50 mM $MgCl_2$ for 10 minutes, followed by additional washing in PBS and dehydration in an ethanol series. When preparing tissue sections for in situ hybridization, pretreatment of the cells with either hot (50–80°C) 1 M NaSCN or hot distilled water for 10 minutes is required to ensure good penetration of the probes. After this, the slides are washed briefly in distilled water and then immediately incubated in pepsin (4.0 mg/ml in 0.2 N HCl, 37°C, 5–20 minutes), followed by rinsing in distilled water and air-drying.

*Procedure for Simultaneous Analysis:* APAAP, IP, or SBB staining derived from immunophenotyping and NISH procedures is not removed by methanol-acetic acid, and the positive cells retain their color during the proteolytic digestion and in situ hybridization procedures. The slides are treated with pepsin and/or NaSCN as with sequential analysis, and the digestion is interrupted by rinsing in distilled water (three times, 3–5 minutes each). The slides are air-dried for at least 30 minutes before in situ hybridization. The pepsin, HCl, or NaSCN treatments as such do not destroy or reduce the color of the immunopositive cells [2, 4, 28, 52–54].

*Comments:* If the NISH detection system is an enzymatic one and based on the horseradish peroxidase enzyme, some cells (e.g., bone marrow cells) are treated with 3% (v/v) $H_2O_2$ in methanol for 30 minutes [52]. For removal of the cytoplasm, also other proteases, such as proteinase K, can be used [53, 55].

**NISH**

*Procedure for Repeat-sequence Probes in Studies of Interphase and/or Metaphase Cells:* The hybridization mixture for repeat-sequence probes consists of 50–60% formamide (BRL), 2.5–10% dextran sulfate (Sigma), 2 × SSC, 0.5 mg/ml herring sperm (Sigma), and 0.001–0.004 μg/μl of the labeled probe. For probe labeling, see *Comments* below. The hybridization mixture is applied to each slide and covered with a glass coverslip. The coverslips are sealed with Fixogum rubber solution (Marabuwerke GmbH & CO., Tamm/Württ., Germany). The probe and the target DNA are usually denatured together by incubating the slides at 70–75°C for 2–5 minutes. In studies of metaphase chromosomes, however, the probe and the target DNA are often denatured separately. This is performed by incubating the slides without the hybridization mixture in 70% formamide-2 × SSC at 70°C for 2 minutes and dehydrating them at 4°C. The probe DNA is denatured separately by incubation at 95°C for 5 minutes. Hybridizations are carried out at 37–42°C for 12–16 hours. After hybridization, the slides are washed successively (5 minutes each) in 50–65% formamide-2 × SSC (pH 7.0), 2 × SSC, and 0.1 × SSC at 44°C, and briefly in 1 × PBS before enzymatic detection, or in 4 × SSC-0.05% Tween 20 before fluorescence detection. The detection is performed as described below [26, 52, 55].

*Comments:* There is no general recipe for nonradioactive in situ hybridization and washing conditions. Nevertheless, protocols reported in the literature have many common features which are also valid for the MAC. For the effects of various components and conditions on hybridization, the reader is referred to Hopman et al. [56].

Probes are nowadays commercially available (e.g., through the American Type Culture Collection, Rockville, MD).

The probes are labeled by standard nick-translation [57, 58] with biotin-11-dUTP (Bethesda Research Laboratories; Sigma), biotin-16-dUTP (Boehringer Mannheim), biotin-14-dATP (Sigma), or digoxigenin-11-dUTP (Boehringer Mannheim) according to the instructions of the nick-translation kit manufacturer (Nick Translation Kit, BRL). Dot blots are used to monitor the labeling of probes as described by Seibl et al. [59]. Also, kits which include labeled probes and reagents for NISH detection are commercially available (e.g., Imagenetics, Naperville, IL).

*Procedure for Chromosomal In Situ Suppression (CISS) Hybridization with Whole Chromosome Probes:* Before CISS hybridization with a whole chromosome probe, the labeled probe (1 μg) is precipitated in ethanol together with human genomic DNA (2 μg) and herring sperm DNA (10 μg). The precipitated probe (1 μg) is then mixed with 10 μl of deionized formamide at 37°C, followed by addition of 10 μl of 20% dextran sulfate-2 × SSC. The probe is denatured at 75°C for 5 minutes. The probe mixture is then incubated at 37°C for 20 minutes (preannealing). The target DNA is denatured by incubating the slides in 70% formamide-2 × SSC at 75°C for 3 minutes, followed by dehydration on ice. After air-drying the slides, the probe mixture is pipetted on each slide. Hybridization is carried out at 37°C for 12–48 hours. After hybridization, the slides are washed three times (5 minutes each) in 50% formamide-2 × SSC (pH 7.0) at 44°C, three times (5 minutes each) in 0.1 × SSC at 58°C, and briefly in 4 × SSC-0.05% Tween 20 before fluorescent detection of the hybrids [28, 60–62].

*Comments:* The probes are labeled as described above. Commercial kits for chromosome painting are also available (Whole Chromosome Painting System, BRL).

*Procedures for indirect enzymatic and fluorescent detection systems:* Hybridizations with repeat-sequence probes are visualized by indirect enzymatic or fluorescent detection. Hybridizations with whole chromosome probes are detected by indirect fluorescent detection methods.

For indirect enzymatic detection of biotinylated hybrids, mouse anti-biotin antibody (Sigma) and peroxidase-conjugated rabbit anti-mouse antibodies (Sigma) are used. To reduce background staining, the slides are first incubated with PBS containing 2% normal rabbit serum (NRS; Vector) and 0.05% Tween 20 at 37°C for 30 minutes, followed by successive incubation with mouse anti-biotin antibody 1:100 and peroxidase-conjugated rabbit anti-mouse antibody 1:80 (Vector) diluted in PBS-0.05% Tween 20 containing 2% NRS at 37°C for 45 minutes. Each incubation step is followed by washing the slides in PBS-0.05% Tween 20 three times (5 minutes each) at room temperature. For peroxidase staining, the slides are incubated with 0.5–1 mg/ml diaminobenzidine tetrahydrochloride (DAB; Sigma) in PBS-0.1 M imidazole (pH 7.7) containing 0.001% $H_2O_2$ at 33–37°C for 5–10 minutes. The slides are rinsed three times for 5 minutes in distilled water, and the cells are counterstained with hematoxylin (Merck) for 1–3 minutes to visualize the nuclei.

Peroxidase-conjugated streptavidin (Vector) can also be used to detect biotinylated hybrids. The slides are incubated at 37°C for 60 minutes in peroxidase-conjugated streptavidin (Vector) diluted 1:200 in PBS containing 5% dry milk and 0.1% Triton X-100. The slides are washed at room temperature twice for 15 minutes in PBS containing 0.1% Tween 20 and once in PBS for 5 minutes. For peroxidase staining, the slides are incubated with 0.5 mg/ml DAB in PBS containing 0.001% $H_2O_2$ at room temperature for 5 minutes. The slides are washed in distilled water. For signal amplification, the slides are incubated in 2.5 mM $NaAuCl_4$ (pH 2.3) for 5 minutes at room temperature, washed in distilled water for 5 minutes, in 0.1 M $Na_2SO_4$ for 5 minutes, and in distilled water for 5 minutes. Silver is precipitated at the sites of probe hybridization by incubating the slides in a silver reagent consisting of 0.24 M $Na_2CO_3$, 0.01 M $NH_4NO_3$, 6 mM $AgNO_3$, 1.5 mM dodecatungstosilicic acid, and 0.6 μl/ml 37% formaldehyde solution at room temperature for 2–8 minutes. After silver amplification, the slides are washed in distilled water at room temperature for 15 minutes, stained with hematoxylin (Merck), and finally rinsed in 0.5% $NH_4OH$ before air-drying.

For indirect fluorescent detection of biotinylated hybrids, the avidin-FITC (fluorescein isothiocyanate, Vector) or TRITC (Vector) detection system is employed. The slides are first incubated in 4 × SSC containing 5% bovine serum albumin (BSA, Boehringer Mannheim) and 0.05% Tween 20 at 37°C for 30 minutes, followed by incubation with FITC- or TRITC-conjugated avidin (1:200/1:1000). The signals are amplified by incubation with biotinylated goat anti-avidin (1:200, Vector) followed by incubation with FITC- or TRITC-conjugated (1:200/1:1000). The antibodies are diluted in 4 × SSC containing 5% BSA and 0.05% Tween 20. Each incubation step is followed by washing the slides three times for 5 minutes each in 4 × SSC-5% BSA-0.05% Tween 20 at 44°C. The slides are counterstained with 1 mg/ml propidium iodide (Sigma) and/or 0.2 mg/ml 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI, Sigma) and mounted in fluorescence antifading reagent consisting of 2% p-phenylenediamine dihydrochloride in PBS:glycerol (1:9, v/v).

With double-target, two-color fluorescence in situ hybridization, the biotinylated probes are detected by avidin-TRITC and the digoxigenin-labeled ones by FITC-conjugated monoclonal antibodies. There are three incubation steps after the post-hybridization washes, the first containing monoclonal mouse anti-digoxigenin antibody (1:500; Sigma) and TRITC-conjugated avidin (1:1000) in 4 × SSC-5% BSA-0.05% Tween 20; the second FITC-conjugated sheep anti-mouse antibody (1:200; Sigma) and biotinylated goat anti-avidin (1:200, Vector), and the third FITC-conjugated donkey anti-sheep antibody (1:200, Sigma) and TRITC-conjugated avidin (1:1000). Each incubation step is followed by washing the slides three times for 5 minutes in 4 × SSC-5% BSA-0.05% Tween 20 at 37°C. The slides are counterstained with DAPI and mounted

in fluorescence antifading buffer as described above [27, 52, 55, 63].

**Analysis of Immunophenotype and Hybridization Signals**

In sequential MAC, for analysis of hybridization signals in immunophenotyped cells, the cells have to be relocalized and compared with photographs taken before NISH. In simultaneous MAC, the immunophenotype and hybridization signals are analyzed at the same time. Depending on the detection systems applied, the cells are examined with a light microscope, phase-contrast microscope (for single fluorescence hybridizations), or a fluorescence microscope equipped with a double band-pass filter combination (Omega) (double fluorescence hybridizations). From 200–1000 cells per sample are analyzed [28, 64].

*Comments:* The following criteria by Hopman et al. [27] are used in evaluating hybridization signals: (1) only cells with good morphology are analyzed; (2) overlapping cells are not analyzed; (3) unspecific signals at minor binding sites are not scored.

Several other versions of our combined immunophenotyping and NISH method have been published for characterization of tumor cells. In these methods, the cells in suspension or fixed on slides are characterized simultaneously by immunofluorescence and fluorescence in situ hybridization (FISH). Most of these methods omit protease treatment prior to hybridization.

Van den Berg et al. [7] and Kibbelaar et al. [8] described a technique in which mononuclear cells in suspension are first stained for membrane antigens by TRITC- or FITC-conjugated monoclonal antibodies, and then spun down onto slides and fixed with methanol:acetic (3:1) acid and 4% formaldehyde. These procedures do not include protease treatment before hybridization, and it should be noted that both the final concentration of formamide (26%) and the temperature (37°C) in the method described by van den Berg et al. [7] are low compared with most other methods reported. Hybridization reactions in this method are visualized using avidin conjugated to FITC, TRITC, or Texas Red.

Weber-Matthiesen et al. [9] described fluorescence immunophenotyping combined with FISH with centromere-specific probes on frozen cytospin slides that are fixed with acetone after thawing. This method involves mild proteinase K treatment (0.5 μg/ml). Weber-Matthiesen et al. [10] also described a method called FICTION (Fluorescence-Immunophenotyping and interphase Cytogenetics as a Tool for Investigation Of Neoplasms), which is a combination of interphase cytogenetics and immunophenotyping on cryostat sections. Sections are first fixed in acetone at room temperature and then immunostained with monoclonal antibodies using fluorescent immunostaining. After immunophenotyping, the specimens are fixed first in cold methanol:acetic acid (3:1) and then in 1% paraformaldehyde. Hybridization reactions are visualized using avidin-FITC. An advanced FICTION method with three-color staining for simultaneous analysis of the immunophenotype and the numbers of two different chromosomes was recently presented by Weber-Matthiesen et al. [11].

Price et al. [12] presented a dual-fluorescence method using the observations of Murdoch et al. [65] and Ziomek et al. [66] that the alkaline phosphatase-Fast Red reaction product of the enzymatic APAAP immunophenotyping method produces a bright red fluorescence that is visible with both FITC and TRITC filters. In the method of Price et al. [12], cells on cytospin slides are first stained with monoclonal antibodies by a three-step enzymatic APAAP immunophenotyping method to determine cell lineage. Alkaline phosphatase-Fast Red is used as a substrate. Hybridization is detected using avidin-FITC.

Strehl and Ambros [13] used FISH combined with immunohistochemistry for detection of chromosome 1 aberrations in neuroblastoma. The cytospin slides they used were stored at −20°C until further processing. After thawing the slides were fixed with 4% paraformaldehyde/1% methanol in 1 × PBS for 10–15 minutes at 4°C, 96% ethanol, methanol, and acetone, each for 20 minutes at 4°C, and with acetone/methanol (2 + 1) for 20 minutes at −20°C. According to them, the slides could be stored for up to 6 months without any adverse effects.

Combined immunophenotyping and NISH have also been used in viral studies to determine the cell types infected [67, 68]. Mullink et al. [67] performed combined immunostaining and NISH on cryostat sections, cytologic preparations, and paraffin sections, using acetone as fixative. According to Mullink et al. [67], the best results were obtained when immunoperoxidase staining with DAB-$H_2O_2$ was followed directly by NISH.

## SIMULTANEOUS VERSUS SEQUENTIAL ANALYSIS OF PHENOTYPE AND GENOTYPE

As mentioned above, the MAC allows both simultaneous and sequential analyses of immunophenotype and hybridization signals. Figure 1 shows that simultaneous enzymatic IP or APAAP immunophenotyping and enzymatic in situ hybridization signal detection provide accurate characterization of cell phenotypes and hybridization signals. In comparison to fluorescent methods, the enzymatic preparations are permanent and easy to photograph. Even combinations of enzymatic immunophenotyping and fluorescence signal detection can be successfully set up. Furthermore, SBB staining and fluorescence signals can be observed simultaneously (Fig. 3).

In sequential analysis, the cells are usually photographed before in situ hybridization, relocalized after the hybridization, and photographed again. Finally, the findings in the two steps are compared. This system yields accurate histology and/or cellular morphology (Fig. 2). For example, in the detection of basophilic granulocytes, the sequential system is superior, as the in situ hybridization procedure destroys the basophilic granulation [69]. Often reliable histologic analysis is also impossible after in situ hybridization. The sequential method is nevertheless slow, and thus simultaneous analysis is the procedure of choice whenever morphologic/histologic characteristics are of secondary interest.

## CONCLUSIONS

The MAC method is a package of techniques yielding, when

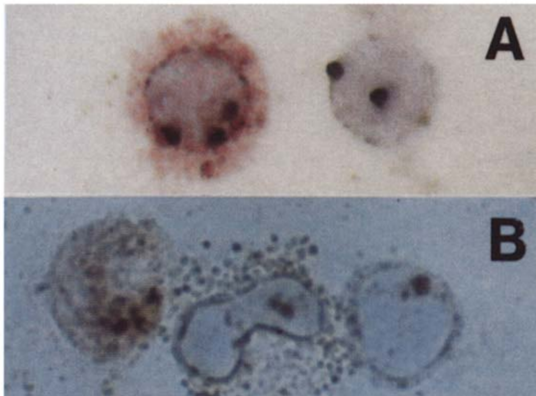


**Figure 2** Simultaneous analysis of immunophenotype and hybridization signals. Cytospin preparations from a male patient having chronic myeloid leukemia (A) and a male patient having acute lymphoid leukemia (B) were immunostained by the alkaline phosphatase anti-alkaline phosphatase technique (APAAP) with CD56 (NK cell)-specific antibody and by the immunoperoxidase technique (IP) and TdT (terminal deoxynucleotidyl transferase)-specific antibody. For in situ hybridization 1- and X-specific centromeric probes, respectively, were used. The positive cells (red and brown) show one extra signal, indicating the abnormality, whereas the negative cells have the normal signal number. Note that in (B) the eosinophilic granulocyte can be recognized according to nuclear morphology and big granulation. However, an accurate morphologic analysis can be done only after sequential analysis, as shown in the next figure.

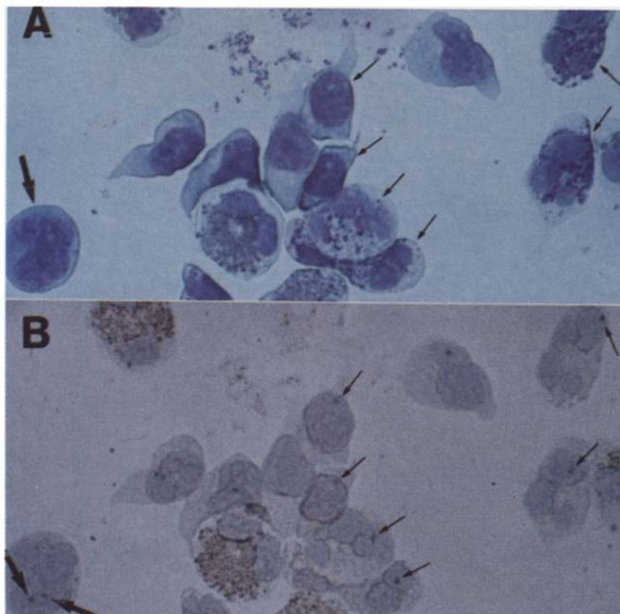


**Figure 3** Sequential analysis of cell morphology and hybridization signals. Cytospin preparation from a male patient having acute lymphoid leukemia, eosinophilia, and chromosome X gain was stained with May-Grünwald Giemsa (A) followed by in situ hybridization with an X-specific centromeric probe (B). Small arrows (A) indicate that basophilic granulocytes (violet) and small lymphocytes have only one signal, whereas a blast cell (large arrow) shows two signals, indicating the abnormality.

all the subtechniques are "on," profound simultaneous information about phenotype (cell morphology, features of cytoplasm and granulation, immunophenotype), genotype (chromosome aberrations, gene/DNA deletions), proliferation kinetics, and clastogenesis on a single mitotic or interphase cell (Figs. 1–5). The MAC method is made especially useful by the possibility of employing only those subtechniques that are relevant to the question being studied and which are supported by available equipment and know-how. Thus, the MAC is well suited not only for use in cytogenetic establishments, but also in hematology and pathology laboratories.

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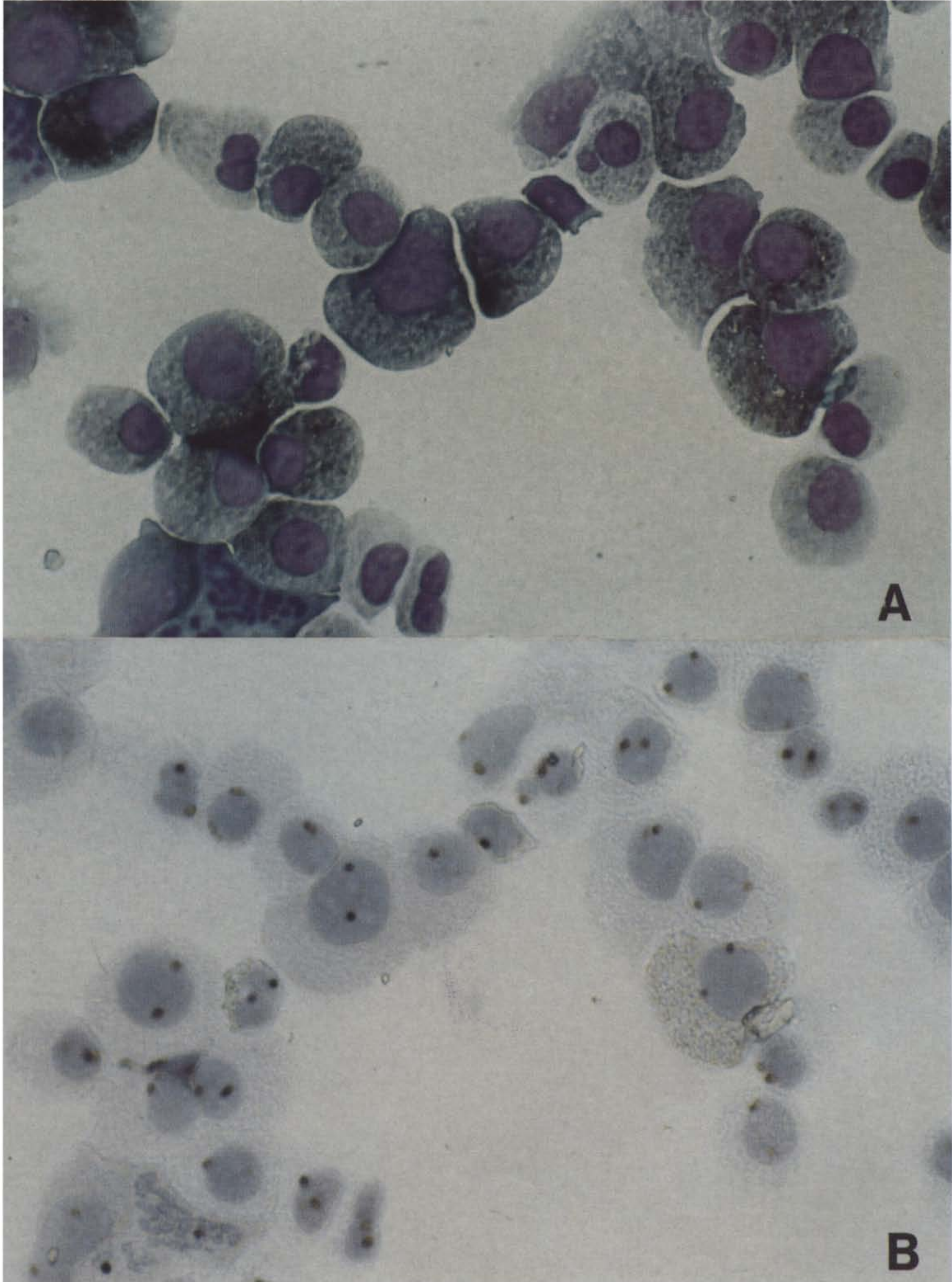


**Figure 4** Simultaneous sequential analysis of Sudan black B staining and hybridization signals. Cytospin preparation from normal bone marrow was stained by Sudan black B (SBB) (A), followed by in situ hybridization with chromosome 1-specific centromeric probe (B). Both positive (black) and negative (grey) cells have two hybridization signals.

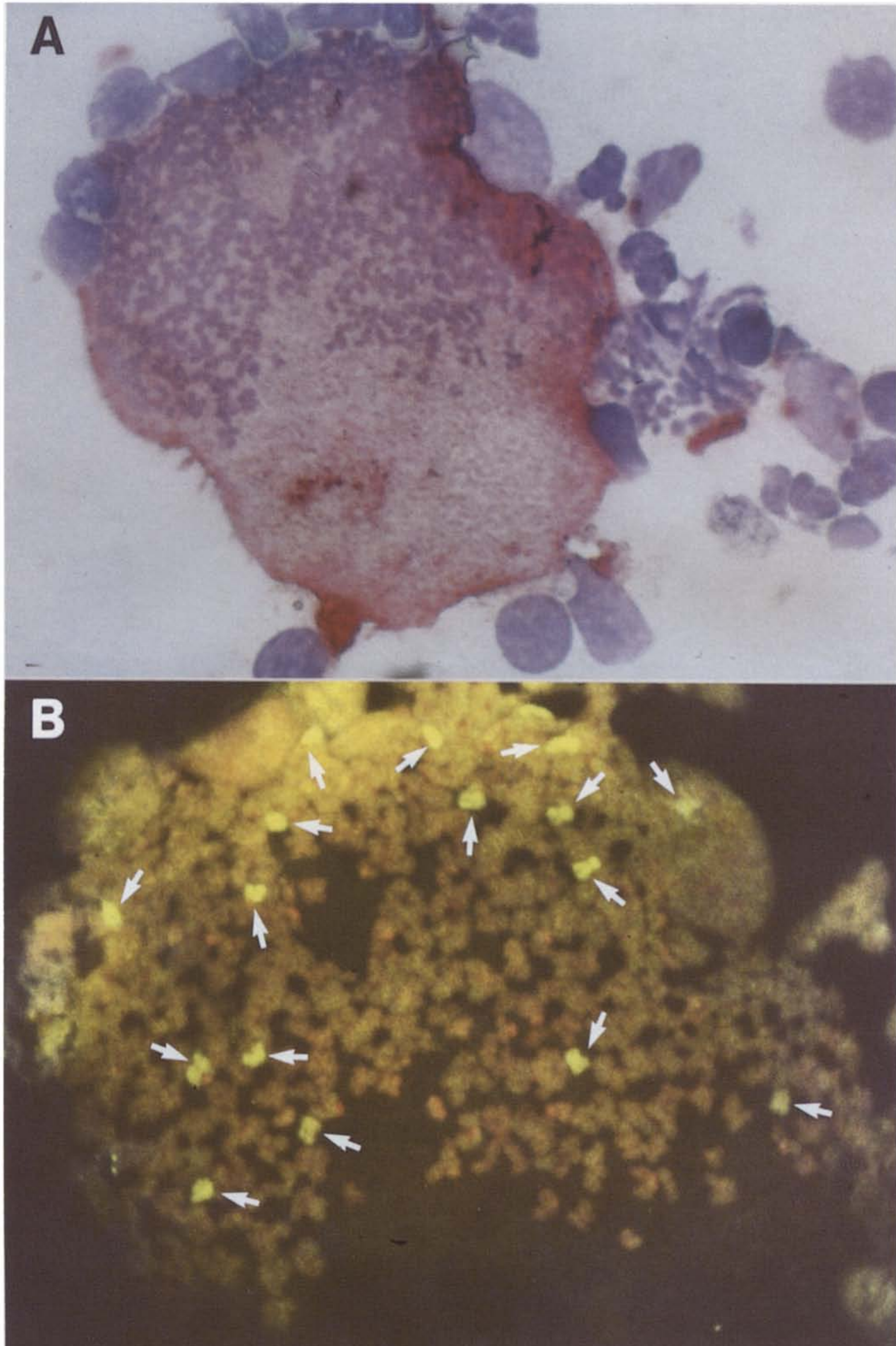


**Figure 5** Immunophenotyping and chromosome painting. Cytospin preparation from a patient with polycythemia vera was immunostained by APAAP with CD61 (megakaryocyte)-specific antibody (A) followed by CISS hybridization with chromosome 20-specific library probe (B). The APAAP-positive octoploid megakaryocytic metaphase cell has 16 copies of chromosome 20.

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