

TECHNICAL NOTE

Improved Resolution of Flow Cytometric Measurements of Hoechst- and Chromomycin-A3-Stained Human Chromosomes After Addition of Citrate and Sulfite¹

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The resolution of bivariate flow karyotypes of human chromosomes stained with Hoechst 33258 and chromomycin A3 can be increased by adding sodium citrate and sodium sulfite to the chromosomes shortly before measurement. A flow karyotype of a patient with chronic myelocytic leukemia is shown to il-

lustrate that the addition of these compounds allows high-resolution measurements to be made and evaluated reliably from clinical samples.

Key terms: Flow karyotype, chronic myeloid leukemia

Human chromosomes stained with the fluorescent dyes Hoechst 33258 and chromomycin A3 can be analyzed for their relative DNA content and base pair composition by flow cytometry. This approach has been used to detect and diagnose karyotypic aberrations such as aneuploidies and chromosomal rearrangements (3,5). The practical value of chromosome analysis by flow cytometry, or flow karyotyping, depends on the reliability of the methods for preparing chromosome suspensions from clinically relevant samples. Much effort has been put into improving procedures for chromosome isolation. Several methods are available for preparing chromosomes from fibroblast cultures, amniocytic cell cultures, and cultures of peripheral blood lymphocytes (1,4,7,8,9,12).

We have observed that the resolution of a flow karyotype is also greatly influenced by the treatment of the chromosomes after they have been obtained in suspension. Manipulation of the free divalent cation concentration by the addition of chelating agents changes the compaction of DNA and modifies the affinity of fluorescent DNA dyes (9,10). Agents that control the redox state of the chromosome suspension can be expected to affect the degree of photo-bleaching that occurs during measurement. We report here that the addition of two such substances (sodium citrate and sodium sulfite) shortly before chromosomes are measured can significantly improve the quality of a flow karyotype.

MATERIALS AND METHODS

Chromosomes are prepared from cells that have accumulated in mitosis through a colcemid block (12h, 0.1 µg/ml colcemid). The cells are swollen in a low ionic strength buffer. After swelling, the cell membranes are mechanically sheared in the presence of a detergent. The isolation buffer contains ingredients that stabilize the chromosomes after they have been released from the cells. In a buffer described by van den Engh et al. (8,9), Mg²⁺ ions serve to stabilize the chromosomes (50 mM KCl, 10 mM MgSO₄, 3 mM dithiothreitol, 5 mM HEPES, pH 8.0, 0.25% Triton X-100). An alternative procedure employs polyamines to stabilize the chromosomes (30 mM KCl, 20 mM NaCl, 14 mM 3-mercaptoethanol, 15 mM Tris-HCl, pH 7.2, 2 mM EDTA, 0.5 mM EGTA, 0.2 mM spermine, 0.5 mM spermidine, 0.1% digitonin) (7). The chromosomes are stained with Hoechst 33258 (preference for A-T base pairs) and chromomycin A3 (preference for C-G base pairs). Chromomycin requires divalent cations for binding to DNA (11). In the polyamine pro-

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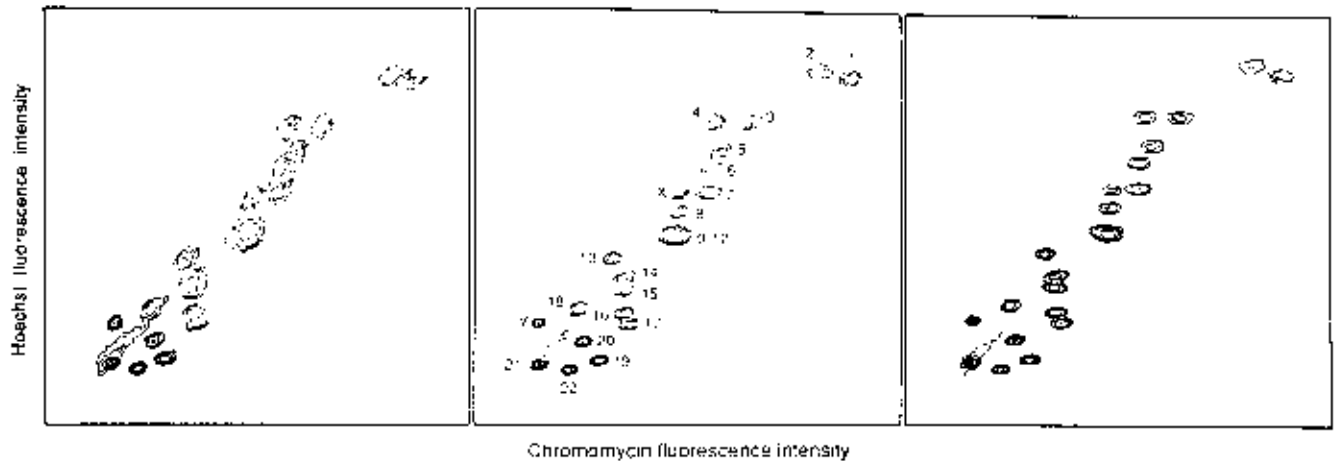


FIG. 1. Bivariate flow karyotype of chromosomes from a karyotypically normal male human fibroblast cell line (HSP-4, kindly provided by L.S. Cram, Los Alamos) isolated in the presence of $MgSO_4$ (9). The chromosomes have been stained with $0.8 \mu M$ Hoechst 33258 and 17

μM chromomycin A3. The left panel shows the distribution of untreated chromosomes. The middle panel shows the preparation after addition of 10 mM Na-citrate. The right panel shows the karyotype of the same suspension after further addition of 25 mM Na-sulfite.

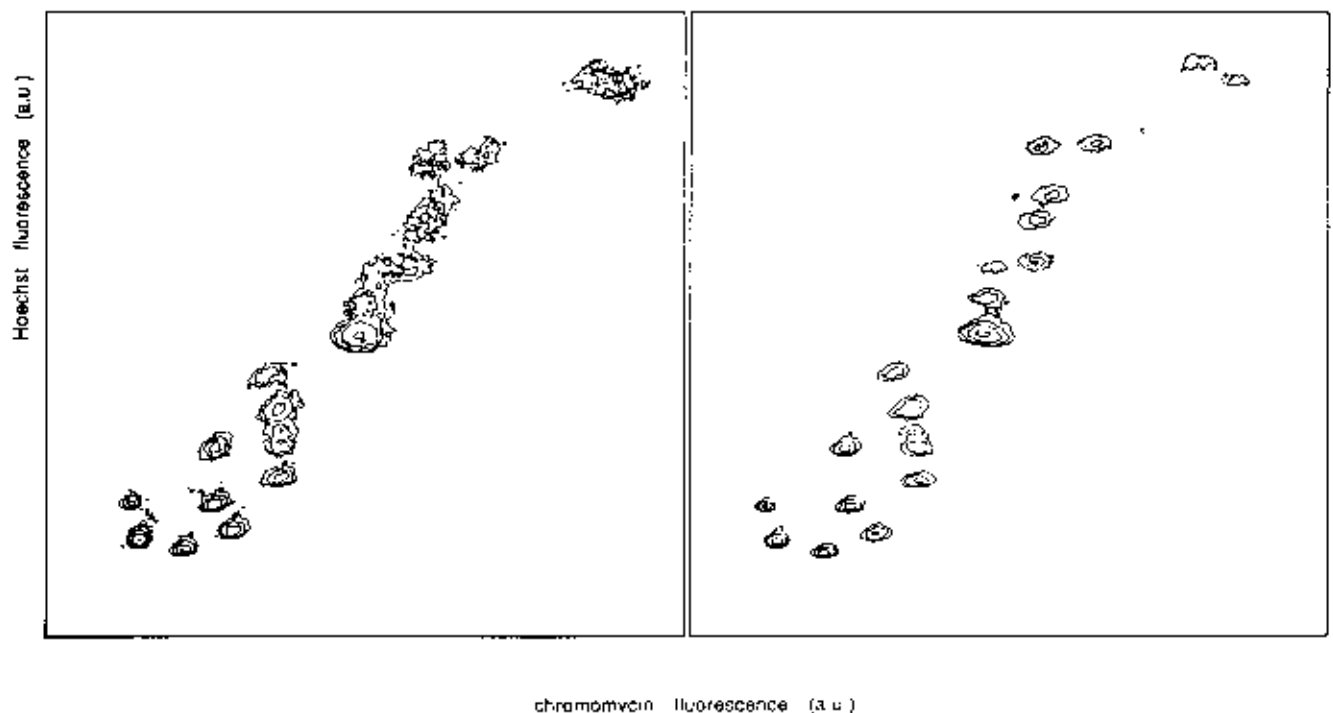


FIG. 2. Bivariate flow karyotype of chromosomes of a karyotypically normal male human lymphoblast cell line (GM2184A, Human Genetic Mutant Cell Repository, Camden, NJ) isolated in the presence of polyamines (7). The chromosomes have been stained with $2.6 \mu M$ Hoechst 33258 and $55 \mu M$ chromomycin A3. The chromomycin solution was

made in $MgCl_2$ -containing buffer (82 mM Na_2HPO_4 , 9 mM citric acid, 25 mM $MgCl_2$, pH 7.0), resulting in a final concentration of 0.3 mM Mg^{2+} after staining. The left panel shows the distribution of untreated chromosomes. The distribution at the right represents the suspension after addition of 10 mM Na-citrate and 25 mM Na-sulfite.

cedure, Mg^{2+} ions are added to the chromosome suspension with the stain. To improve karyotype resolution, Na-citrate and Na-sulfite were added to the chromosomes 15–30 minutes before measurement from a $10\times$ stock solution (100 mM Na-citrate, 250 mM Na-sulfite,

pH 10). The final pH of the stained chromosomes in $MgSO_4$ and polyamine was 8.1 and 7.4, respectively. The suspensions are analyzed in a dual-beam flow cytometer (2). One laser is tuned to emit light at 458 nm (400 mW). The second laser emits light in the UV ($351\text{--}364 \text{ nm}$, 1

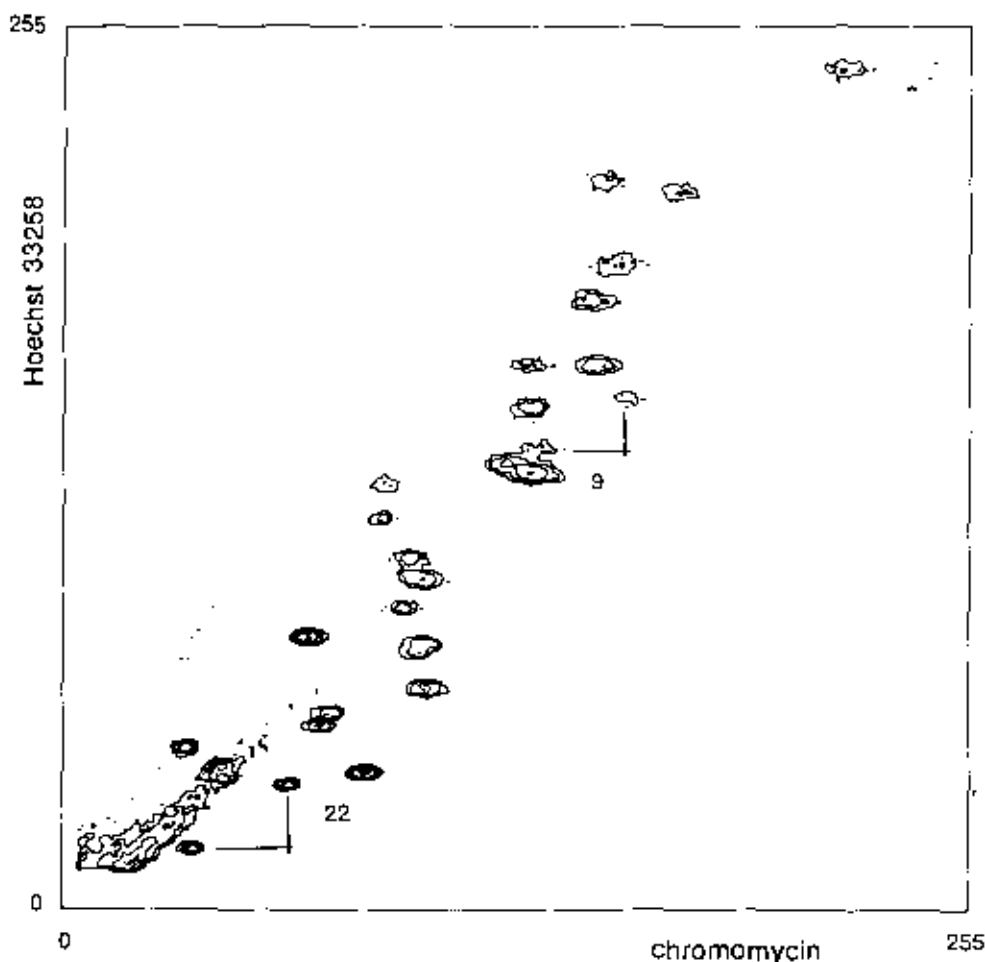


FIG. 3. Flow karyotype of cells from a male patient with chronic myeloid leukemia after addition of Na-citrate and Na-sulfite. The chromosomes were isolated using the $MgSO_4$ procedure. The peak near the origin represents the small derivative 22, or Philadelphia, chro-

somosome. The peak to the right of the 9-12 group represents the derivative 9 chromosome. All other homologs are normal variations that are also present in normal cells of the same patient.

W). The intensities of the fluorescence pulses from the individual chromosomes are processed by a fast data acquisition system (van den Engh and Stokdijk, in preparation). The measurements are stored as a list by a Hewlett-Packard 9000 computer. The flow karyotypes are presented as 256×256 channel bivariate distributions.

RESULTS

Human chromosome suspensions that are measured by dual-beam flow cytometry yield bivariate distributions in which most of the chromosomes can be recognized as well-separated clusters. The peak width and cluster separation can be improved by adding sodium citrate and sodium sulfite to the suspension shortly before analysis. Figures 1 and 2 show bivariate distributions of preparations of human chromosomes before and after the addition of 10 mM Na-citrate and 25 mM Na-sulfite. The chromosomes of Figure 1 were prepared using the $MgSO_4$ procedure. The karyotypes in Figure 2

are from chromosomes prepared using the polyamine method. In both cases, karyotypes measured in the presence of citrate and sulfite show a marked tightening of the peaks. The changes in the coefficients of variation (CV) of the chromosome peaks are summarized in Table 1. The CVs of the Hoechst fluorescence improve from 2-4% to 1-2%. The CVs of the chromomycin fluorescence measurements improve from approximately 4% to 3%. Chromosomes that have overlapping distributions in the absence of citrate and sulfite, such as those in the 14-17 group, usually become clearly separated when the two compounds are added. In the preparation with additives, the 9-12 group also shows some structure indicating that the small differences in DNA content and base composition among these chromosomes are beginning to be resolved. With the low peak CVs after citrate and sulfite addition, the two homologs of many of the human chromosomes can be resolved.

The effects of citrate and sulfite are observed within a few minutes after their addition. Best results are ob-

Table 1
Coefficient of Variation (% of mean) of Selected Chromosome Peaks Before and After Addition of 10 mM Na-Citrate and 25 mM Na-Sulfite^a

	MgSO ₄ isolation		Polyamine isolation	
	Before	After	Before	After
Hoechst fluorescence				
chromosomes 1-6	2.1	1.3	2.0	1.3
chromosomes 13,17	2.9	1.8	2.7	2.1
chromosomes 18,19,20,22,Y	3.5	2.0	3.8	2.8
Chromomycin fluorescence				
chromosomes 1-6	3.5	2.2	3.0	2.7
chromosomes 13,17	4.0	2.4	4.2	3.6
chromosomes 18,19,20,22,Y	3.6	3.4	4.6	4.1

^a The coefficients of variation were determined for various peaks in the flow karyotype. Two univariate Gaussian distributions with equal area and equal variance (representing the two homologs) were fit to each peak using an iterative least squares fitting procedure. When individual chromosome peaks could not be resolved, two peaks were treated as one and fit with four univariate distributions. The CVs were calculated as the variance/mean \times 100%. The CVs displayed here represent the average CV for the chromosomes in each group.

tained after the chromosomes have equilibrated with citrate and sulfite for 15-30 min and persist for several hours. Addition of citrate alone noticeably improves flow karyotype resolution. In contrast, the improvement by sulfite is more subtle and is only observed in the presence of citrate. A similar increase of karyotype resolution is observed when citrate and sulfite are added to chromosomes that have been stained with DAPI instead of Hoechst.

The addition of citrate and sulfite is particularly beneficial in the analysis of chromosome preparations that contain a large amount of small particle debris or chromosome clumps. The tightening of the peaks increases their heights relative to the background continuum of the particles. This improvement may make it possible to derive information from clinical samples that may be otherwise impossible to interpret. An example is given in Figure 3. This flow karyotype shows the chromosomes of leukemic cells from a patient with chronic myeloid leukemia (CML). The leukemic cells were cultured for 5 d in the presence of PHA-stimulated leukocyte-conditioned medium. This medium contains growth stimulators that are required by the leukemic cells. The culture was set up at the British Columbia Cancer Research Centre in Vancouver, sent to Livermore by overnight mail, and cultured another 24 h before colcemid addition. The culture had a low mitotic index (approximately 4%). The resulting chromosome preparation contained nuclei, dead cells and small fluorescent debris and, without citrate and sulfite addition, was unsuitable for flow karyotyping. After citrate and sulfite addition, the peak CVs were reduced to approximately 1-2%, and the chromosomes became well resolved above the underlying debris distribution. Homolog differences of chromosomes 1, 13, 14, 15, 16, 19, and 21 became distinguishable. The same homolog differences were observed in a chromosome preparation of an EBV-transformed lymphoblast culture of normal cells from the same patient (data not

shown). Two peaks, which were not seen in the flow karyotype of the normal cells, were present in the karyotype of the leukemic cells. These peaks were produced by the two derived chromosomes that resulted from the (9;22) translocation that is diagnostic for CML (6).

DISCUSSION

The addition of citrate and sulfite has significantly increased our ability to produce and evaluate flow karyotypes from amniocyte cultures, peripheral blood cultures, and leukemic cell cultures. Karyotype quality as observed in Figures 1 and 2 is routine. The resolution of these karyotypes seems to be determined by the precision of the measuring equipment rather than the quality of the stained chromosome preparation. This statement is supported by the following observations:

1. The alignment of the optical components of the cytometer is critical. During measurements, a gradual loss of resolution is often observed. Readjustment of the flow chamber and illumination optics restores the flow karyotype quality.

2. Flow karyotype resolution is related to the intensity of the illumination beams. The measurements described here were made with 400 mW of 457-nm excitation light and 1 W of UV. Flow karyotype resolution decreases at lower light intensities.

The mechanisms of the citrate and sulfite effects are unclear. Citrate has previously been shown to affect DNA:dye interactions and DNA compaction (10). Addition of citrate changes the absolute fluorescence intensities of Hoechst (increases) and chromomycin (decreases). It also reduces the amount of energy transfer between the two dyes (10). Citrate may exert its activity through its moderate chelating properties. Its affinity for divalent cations may be strong enough to affect dye-DNA binding, but not strong enough to negatively affect

DNA stability. The effect of sulfite may be due to the reduction of oxygen and/or other electron acceptors that may cause fluorochrome bleaching. These effects can be expected to be small and, therefore, would be noticeable only under ideal staining and measuring conditions. Preliminary evidence suggests that addition of citrate and/or sulfite may also be beneficial for other types of flow cytometric measurements. Considering the dramatic improvements that have been observed in flow karyotyping, it seems worthwhile to test these compounds in other assays in which histogram resolution or signal intensity is a problem.

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