

# Chapter 2

## Chromosome Preparation for Myeloid Malignancies

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### Abstract

Many cases of myeloid malignancies are associated with recurring cytogenetic aberrations. Chromosomal analysis can aid in diagnosis, predict prognosis, and disclose subsequent clonal evolution. Three different cell culture methods: direct harvest, nonsynchronized culture, and synchronized culture are usually prepared if the nucleated cell counts in marrow blood are sufficient. Synchronized culture is the first choice of method in myeloid malignancies, whereas the direct method can be omitted if the cell count is low. The aseptic culture technique is strictly followed until harvesting procedure. For synchronized culture, uridine and fluorodeoxyuridine are added as blocking reagents and released by thymidine on the following day. Harvesting steps of the cultures involved colcemid exposure, hypotonic treatment, and Carnoy's fixation. The cells are then ready for slide making and banding for chromosomal analysis.

**Key words** Myeloid malignancies, Chromosome preparation, Cytogenetic culture, Synchronization culture, Metaphase harvesting

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### 1 Introduction

Myeloid malignancies include acute myeloid leukemia (AML), myeloproliferative neoplasm (MPN), and myelodysplastic syndrome (MDS). The new classification of hematopoietic and lymphoid neoplasms was first introduced in 2001 by the World Health Organization (WHO), the Society for Hematopathology and the European Association for Haematopathology [1, 2]. In addition to the assessment on morphology and cytochemistry of the neoplastic cells as adopted by the French-American-British (FAB) system for the classification of acute myeloid leukemia (AML) since 1976 [3], the new classification of myeloid neoplasm has incorporated genetic information to establish specific disease entities and predict the prognosis more accurately. Many cases of AML are found to have recurring genetic abnormalities that affect cellular pathways of myeloid cells. In 2008, WHO revised the classification of myeloid neoplasm to provide an updated version based on recent data [4]. Additional chromosomal rearrangements are further updated the category of AML with recurrent genetic

**Table 1**  
**WHO classification of acute myeloid leukemia with recurrent genetic abnormalities [5]**

AML with t(8;21)(q22;q22.1); <i>RUNX1-RUNX1T1</i>
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); <i>CBFB-MYH11</i>
AML with <i>PML-RARA</i>
AML with t(9;11)(p21.3;q23.3); <i>MLLT3-KMT2A</i>
AML with t(6;9)(p23;q34.1); <i>DEK-NUP214</i>
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); <i>GATA2, MECOM</i>
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); <i>RBM15-MKLI</i>
AML with <i>BCR-ABL1</i> (provisional entity)
AML with mutated <i>NPM1</i>
AML with biallelic mutations of <i>CEBPA</i>
AML with mutated <i>RUNX1</i> (provisional entity)

abnormalities in 2016 revision (Table 1) [5]. Cytogenetic analysis of bone marrow cells is important during initial evaluation for diagnosis and prediction of prognosis. Patients with AML harboring t(15;17)(q22;q21), t(8;21)(q22;q22), and inv(16)(p13.1q22)/t(16;16)(p13.1;q22) are associated with relatively favorable outcomes, whereas those with inv(3)(q21q26.2)/t(3;3)(q21;q26.2), MLL rearrangement (except t(9;11)(p22;q23)), deletion of 5q, monosomies of chromosome 5 and/or 7, or complex karyotypes are associated with poorer prognoses [6].

Bone marrow aspirate in heparin or in culture medium should be sent to the laboratory as soon as possible without delay at room temperature. White cell count is adjusted to  $1 \times 10^6$  cells/mL of culture medium. At least two different culture methods, nonsynchronized and synchronized, are set up if white cell count is adequate. If insufficient cells are present in the specimen, synchronized culture is preferred for myeloid malignancy.

The principle of cell cycle synchronization is to block the cells at the synthesis (S) phase causing an accumulation of many cells at this particular stage and release the cells on the next day so that many cells enter mitosis at approximately the same time. Better banding quality and longer chromosomes can thus be achieved. Fluorodeoxyuridine (FdU) and uridine prevent the synthesis of thymidine by blocking the action of thymidylate synthetase, an important enzyme in the production of thymidine. Cells are then blocked in S-phase in the cell cycle. These blocking reagents are usually added 16–20 h before harvesting. On the next morning, thymidine is added to release the block and the cells can resume their cell

cycles. The block is removed 4–5 h before harvesting to let the cells go through the remaining cell cycle and enter into mitosis.

Harvesting begins with the addition of mitotic spindle inhibitor that depolymerizes the microtubules, which make up the spindle fiber. The chromosomes are freed from the metaphase plate without the spindle fiber, thus allowing them to float freely within the cytoplasm. As a result, the cells are arrested at metaphase. Colcemid is an analog of colchicine which is less toxic and is the most commonly used mitotic inhibitor nowadays. After the mitotic arrest, cells are centrifuged and resuspended in hypotonic solution. This hypotonic treatment causes swelling and lysis of the red blood cells, which facilitates better metaphase spreading. The final step involves fixing the cells using freshly prepared Carnoy's fixative. The cells become dehydrated while the cell membrane is hardened in the fixation process. The cells are washed with Carnoy's fixative until a clear solution is obtained. Fixed cells can be kept in  $-20^{\circ}\text{C}$  for slide making and banding over years [7–9].

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## 2 Materials

### 2.1 Equipments

1. Class 2 biological safety cabinet (*see Note 1*).
2. Humidified  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  incubator (*see Note 2*).
3. Bench-top automated cell counter (*see Note 3*).
4. Centrifuge.
5. Water bath.
6. Sterile 25-cm<sup>2</sup> flask with ventilation cap (*see Note 2*).
7. Sterile 15-mL centrifuge tubes.
8. Sterile transfer pipettes.

### 2.2 Reagents

All containers and distilled water used in reagents 1–5 should be sterile.

1. Growth medium: 1000 mL of RPMI 1640 medium (*see Note 4*), 180 mL of fetal calf serum, 12 mL of penicillin & streptomycin, 12 mL of preservative-free heparin (1000 IU/mL), 12 mL of L-glutamine. Store aliquots at  $-20^{\circ}\text{C}$  for 2 months.
2. Chang medium BMC. Store aliquots at  $-20^{\circ}\text{C}$  (*see Note 5*).
3. Working fluorodeoxyuridine (FdU): Dissolve 10 mg of FdU (M.W. 246.2) in 40 mL of distilled water as super-stock (1 mM). Pass through 0.45  $\mu\text{m}$  filter to sterilize and store 1 mL aliquots at  $-20^{\circ}\text{C}$  for 2 years. Add 9 mL of distilled water to 1 mL of super-stock as stock solution (100  $\mu\text{M}$ ). Pass through 0.45  $\mu\text{m}$  filter to sterilize and store 1 mL aliquots at  $-20^{\circ}\text{C}$  for 2 years. Add 9 mL of distilled water to 1 mL of

stock solution as 10  $\mu$ M working solution. Store at 4 °C for 1 month.

4. Working uridine: Dissolve 97.7 mg of uridine (M.W. 244.2) in 100 mL of distilled water as stock solution (4 mM). Pass through 0.45  $\mu$ m filter to sterilize and store 1 mL aliquots at –20 °C for up to 2 years. Add 9 mL of distilled water to 1 mL aliquot as 0.4 mM uridine working solution. Store at 4 °C for 1 month.
5. Working thymidine: Dissolve 24.22 mg of thymidine (M.W. 242.2) in 10 mL of distilled water as stock solution (10 mM). Pass through 0.45  $\mu$ m filter to sterilize and store 1 mL aliquots at –20 °C for up to 2 years. Add 9 mL of distilled water to 1 mL aliquot as 1 mM thymidine working solution. Store at 4 °C for 1 month.
6. 1 $\times$  Phosphate buffered saline (PBS): Dissolve 8 g of NaCl, 0.2 g of KCl, 0.92 g of Na<sub>2</sub>HPO<sub>4</sub>, and 0.2 g of KH<sub>2</sub>PO<sub>4</sub> in 1 L of distilled water. Adjust pH to 7.2.
7. Colcemid (KaryoMax, Gibco): 10  $\mu$ g/mL solution (*see Note 6*).
8. 0.054 M (0.4 %) Potassium chloride (KCl): Dissolve 4 g of KCl (M.W. 74.55) in 1 L of distilled water (*see Note 7*).
9. Carnoy's fixative: Freshly prepare 3:1 (v/v) absolute methanol/glacial acetic acid (*see Note 8*).

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### 3 Methods

Carry out all procedures in a Class 2 safety cabinet using the aseptic culture technique until cell harvest. Pre-warm all medium at 37 °C.

#### 3.1 Measurement of Nucleated Cell Count and Cell Washing

1. 1–2 mL of bone marrow aspirate is collected in a preservative-free heparin bottle or in 8 mL of growth medium (*see Note 9*).
2. Note the volume of bone marrow.
3. Mix 50  $\mu$ L of bone marrow with 450  $\mu$ L of saline or culture medium. Measure nucleated cell count of the bone marrow using bench-top cell analyzer.
4. Calculate the nucleated cell in bone marrow as follows:  
white cell count in analyzer ( $10^6$ /mL)  $\times$  10 (dilution factor)  $\times$  volume of bone marrow (mL).
5. Add approximate  $1 \times 10^7$  total nucleated cells to each 10 mL of culture (*see Note 10*). Set up direct harvest, synchronized culture, and nonsynchronized culture if adequate amount of nucleated cells is available (*see Note 11*).

6. Take out appropriate volume of bone marrow into a sterile 15-mL centrifuge tube.
7. Wash the bone marrow with growth medium. Make up the volume to 10 mL and centrifuge at  $200\times g$  for 10 min.
8. After centrifugation, remove supernatant and resuspend the cell pellet in 1 mL.
9. Proceed each tube for direct harvest (*see* Subheading 3.2), nonsynchronized culture (*see* Subheading 3.3), and synchronized culture (*see* Subheading 3.4).

### 3.2 Direct Harvest

1. Add 9 mL of growth medium and 50  $\mu$ L of colcemid.
2. Incubate in 37 °C water bath for 45 min.
3. Proceed with cell harvest (*see* steps 5–14 in Subheading 3.5).

### 3.3 Nonsynchronized Culture

1. Add 5 mL of Chang medium and 4 mL of growth medium.
2. Transfer all the contents to the culture flask.
3. Incubate in 37 °C, 5% CO<sub>2</sub> incubator for 1–3 days.
4. Proceed with cell harvest (*see* Subheading 3.5).

### 3.4 Synchronized Culture

1. Add 5 mL of Chang medium and 4 mL of growth medium.
2. Transfer all the contents to the culture flask.
3. Add 100  $\mu$ L of working FdU and 100  $\mu$ L of working uridine to the culture after at least 2 h incubation preferably to let the cells acclimatize the culture environment. Otherwise, these reagents can be added on the following day.
4. Incubate in 37 °C 5% CO<sub>2</sub> incubator overnight.
5. Add 100  $\mu$ L of working thymidine in the next morning. Incubate for further 5–7 h prior harvesting.
6. Proceed with cell harvest (*see* Subheading 3.5).

### 3.5 Cell Harvest

1. Add 30  $\mu$ L of colcemid to a 15-mL centrifuge tube (*see* Note 12).
2. Transfer culture to the centrifuge tube.
3. Incubate in 37 °C water bath for 30 min.
4. Pre-warm 0.4% KCl to 37 °C.
5. After incubation, centrifuge the culture at  $200\times g$  for 10 min.
6. Discard supernatant and resuspend the pellet.
7. Add pre-warmed 0.4% KCl and top up to 10 mL.
8. Incubate in 37 °C water bath for 16 min.
9. After incubation, add 1 mL of Carnoy's fixative with inverted mixing (*see* Note 13).

10. Centrifuge at  $200\times g$  for 10 min.
11. Discard supernatant.
12. Add 8–10 mL of Carnoy's fixative with inverted mixing and incubate for 10 min at room temperature.
13. Repeat **steps 10–11** at least twice until the suspension appears clear.
14. Store at  $-20^{\circ}\text{C}$  for subsequent slide making and banding.

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## 4 Notes

1. Class 2 biological safety cabinet is characterized by a vertical laminar flow of air, which is filtered through a high efficient hepa filter. It can protect both the worker and specimen from microbial contamination.
2. An open culture system is used in this protocol which allows gaseous exchange between the air inside the flask and the environment within the incubator. It helps to maintain the pH of the culture media between 7.2 and 7.4 by the reaction of sodium bicarbonate in the medium and  $\text{CO}_2$  environment of the incubator. A trough of water is needed to place on the lowest shelf of the incubator to avoid evaporation of the medium. Vented flasks with  $0.2\text{ }\mu\text{m}$  membrane filter are used to allow gaseous interchange and protect from microbial contamination.
3. Dilute the marrow blood with PBS or culture medium before aspirating into the cell counter to avoid clotting of the analyzer. Alternatively, the nucleated cell count can also be achieved manually by using hemocytometer.
4. RPMI 1640 medium was originally developed to culture human leukemic cells in suspension and as a monolayer. It requires supplement with 10 % fetal bovine serum (FBS) and uses a sodium bicarbonate buffer system ( $2.0\text{ g/L}$ ). A 5 %  $\text{CO}_2$  incubator is used to maintain optimum pH for cell growth.
5. Chang medium BMC is intended for use in primary culture of clinical human bone marrow cells for karyotyping. It consists of RPMI medium 1640, FBS, hepes buffer, L-glutamine, giant cell tumor conditioned medium, and gentamicin sulfate (Irvine Scientific).
6. Gibco KaryoMAX colcemid solution is a  $10\text{ }\mu\text{g/mL}$  *N*-desacetyl-*N*-methylocolchicine solution made up in Hanks' balanced salt solution (HBSS). It prevents spindle formation during mitosis, arresting cells in metaphase so that the chromosomes can be separated for cytogenetic studies and in vitro diagnostic procedures. The mechanism of action is similar to that of colchicine, but with lower mammalian toxicity.

7. Alternative hypotonic solutions are 0.075 M KCl, water, 0.4 % sodium citrate, or dilute medium.
8. Carnoy's fixative must be freshly prepared since methanol reacts with acetic acid to form methyl acetate on prolonged standing, which may lead to improper drying and spreading of the chromosomes. They should be kept in air-tight containers to prevent water being absorbed.
9. Fresh bone marrow aspirate, preferably the first portion, should be sent to the laboratory as soon as possible at room temperature. Bone marrow aspirate in EDTA bottle is unsatisfactory specimen as EDTA is toxic to the cells that may not yield viable culture.
10. Too high nucleated cell count in the culture may lead to depletion of nutrients in the medium. Conversely, low cell count in the culture does not grow well and will result in inadequate metaphase available for chromosomal analysis.
11. Synchronized culture is the best choice for myeloid malignancies where direct method usually fails if the cell count is insufficient. In acute promyelocytic leukemia, the abnormality t(15;17) (q22;q21) is usually not present in the direct method.
12. The longer exposure and higher concentration of colcemid produce greater contraction of the chromosome.
13. This step is called pre-fix. If this step is missing, Carnoy's fixative can also be added at **step 12** in Subheading 3.5. However, a few drops of fixative need to be added first with thorough agitation of the cell pellet before adding the rest of fixative to avoid cell clumping. This may require a skillful technique.

## References

1. Jaffe ES, Harris NL, Stein H, Vardiman JW (eds) (2001) World Health Organization classification of tumours. Pathology and genetics of tumours of hematopoietic and lymphoid tissues. IARC, Lyon
2. Vardiman JW, Harris NL, Brunning RD (2002) The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood* 100(7):2292–2302
3. Bennett JM, Catovsky D, Daniel MT et al (1976) Proposals for the classification of the acute leukaemia: French-American-British. Cooperative Group. *Br J Haematol* 33:451–458
4. Swerdlow SH, Campo E, Harris NL et al (eds) (2008) WHO classification of tumours of hematopoietic and lymphoid tissues. IARC, Lyon
5. Arber DA, Orazi A, Hasserjian R et al (2016) The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood* 127(20):2391–2405
6. Grimwade D, Hills RK, Moorman AV et al (2010) Refinement of cytogenetic classification in acute myeloid leukaemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. *Blood* 116(3):354–365
7. Dunn B, Mouchrani P, Keagle M (eds) (2005) The cytogenetic symposia, 2nd edn. Association of Genetic Technologists, Olathe, KS
8. Dunn B, McMorow LE (2008) Cytogenetic study guide. Foundation for Genetic Technology, Lenexa, KS
9. Hopwood VL, Gu J, Zhao M (eds) (2011) The cytogenetic technology program: A comprehensive review in clinical cytogenetics. MD Anderson Cancer Center, Houston, TX

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