

Blood Film Preparation and Staining Procedures

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ABSTRACT

The blood film is one of the world's most widely used laboratory tests for screening, case finding, diagnosis, and monitoring of disease. This article provides guidelines that should enable clinical laboratories to prepare and stain good-quality blood films. Objective criteria have been applied as much as possible, although there remains some "art" in how a blood film is prepared manually. Troubleshooting of fixation and staining artifacts is included in the guidelines. *Lab Hematol.* 2000;6:1-7.

KEY WORDS: Blood film preparation · Staining

INTRODUCTION

The blood film is one of the world's most widely and frequently used tests, and yet there appears to be no comprehensive document that lists requirements, procedures, potential problems, etc., for blood film preparation. Although it is a simple procedure, there are many reasons that as a test it could easily fail or be less effective than it should be. This article was prepared at the request of the Cytometry Panel of the International Council for Standardization in Haematology (ICSH) as a guideline for laboratories. It is intended to give direction and some standardization in the preparation and staining of blood films for morphological evaluation in the clinical laboratory. Microscopic analysis procedures and interpretation are not within the scope of this article.

Methods include state-of-the-art techniques as well as methodologies applicable for laboratories in developing countries. When targets are stated, they are not at the maximum level but are targets that should be attained under all conditions lest diagnostic test quality and therefore patient care be compromised. This is especially important since results obtained from microscopic analy-

sis of blood films are often final and definitive in clinical situations, whether for case finding, diagnosis, or monitoring of disease.

For easier reference use, this article uses a special format to describe the various steps that involve blood film preparation and staining.

BLOOD FILM PREPARATION

Microscope Slides

Slides should be made from the highest purity, corrosion-resistant glass; other material such as plastic is mostly not acceptable. Glass slides typically measure 75 × 25 mm, are approximately 1 mm in thickness, must be flat and free from distortions and ripples, and must be clear and colorless ("water-white"). For fluorescence microscopy, glass that does not show autofluorescence must be used.

It is preferable to use precleaned slides, but at minimum, laboratories must ensure that slides are free from scratches; are clean; are free of dust, lint, and fat (from fingerprints); and are dry. This may mean that in humid environments, slides must be hydration resistant and, after their sealed container is opened, should be kept in a desiccator or container with water-free (methyl) alcohol or a mixture of 3 parts alcohol and 1 part acetone until used.

Slides should always be stored in sealed containers to be opened only immediately before use. They may be plain or have a frosted or coated area for writing. Slides with rounded or beveled edges are safer to use than those with squared edges and reduce the chances of cut or punctured skin and gloves.

Cleaning of Slides

Dirty slides need to be cleaned by soaking in a detergent at 60°C for 15 to 20 minutes, then rinsing in hot water before drying. New slides that need cleaning should be placed in a potassium dichromate cleaning solution (20 g Cr₂K₂O₇ in 100 mL water with 900 mL concentrated H₂SO₄) for 48 hours. This procedure is followed by a thorough rinse in running tap water. The slides should be stored in 95% methanol and carefully wiped clean and dried before use [1].

Blood Film Preparation Methodologies

Typical blood film preparation methodologies are: manual (wedge, coverslip), semi-automated (wedge, spinner), and automated (wedge).

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Wedge Method ("Push")

This method is commonly used in manual as well as in automated and semi-automated environments. When the wedge method is carried out properly, a sufficiently large area is available for microscopic examination: this area shows all cells barely touching or separated from each other (monolayer part). The parts of the film farthest away from the start will be too thin (with morphological alterations as a result), whereas the part proximal to the start of the push will be too thick for microscopy.

Automated devices are capable of providing excellent quality blood films, usually with greater consistency than those obtained by manual methods [2]. The main concern with wedge preparations is the uneven distribution of different cell types [3]. Monocytes (and other large leukocytes) in particular are pushed to the end of the spread film (the "feathered" edge) and to the sides. This leads to a 5% to 10% underestimation of monocyte presence compared with monoclonal antibody-based flow cytometry differential counts (upper normal limit in proportional count is 11% in normal individuals, not 10%, as measured by microscopy) [4].

Coverglass Method ("Pull")

The small coverglass slides cannot be labeled adequately and should therefore be avoided. In addition, the technique itself has a biohazard risk higher than that for the wedge method. The method is considered obsolete and should not be used.

Spun Blood Film

This method has been described as an alternative to wedge methods [5,6]. By spreading blood cells via centrifugal force over large areas, it offers a monolayer of blood cells for microscopic examination. The morphological condition of all cell types in properly prepared spun films is generally excellent, although care has to be taken to avoid the formation of smudge cells [7]. Dilution of blood with isotonic saline has been recommended [8], but excellent preparations are generally possible without this step. There is no evidence that spun blood film preparations result in uneven distribution of cell types [9].

Spinners from the past were among the most hazardous of all laboratory instrumentation because of droplet and aerosol formation and contamination of the spinner's interior by blood. Recently developed spinners have overcome these problems and can be used safely¹ [10].

It is essential to apply exactly the right amount of blood to these spinners to obtain consistent monolayers; therefore, the use of micropipettes rather than capillary tubes is recommended for dispensing blood.

Type of Blood Sample

The two types of blood sample are venous (anticoagulated) and capillary, as obtained by skin puncture (without anticoagulation). There are several published standard documents on devices used for and procedures for specimen collection, and the reader is referred to those texts for details [11-14].

Anticoagulation

Acceptable anticoagulant agents are K₂EDTA or K₃EDTA, sodium citrate, and acid citrate-dextrose (ACD). Heparin is not recommended because of frequently developing platelet clumps that interfere with the morphological interpretation of platelets and platelet count estimates. Heparin also causes the development of a purple/blue hue on stained films.

Sample Storage Effects

Blood samples should be processed as quickly as possible after collection. Significant morphological changes occur on prolonged storage and are time and temperature dependent. Best results are obtained when films are prepared within 2 hours after collection.

Storage temperatures are as follows: short-term (<8 hours): preferably at 4°C, but storage at room temperature is acceptable; long-term: 4°C. Always mix blood samples after prolonged storage by a minimum of 10 complete (180°) inversions.

Capillary Tubes

Plastic (polystyrene or similar) tubes (unbreakable) are recommended for placing blood drops onto the slide. Glass tubes should be avoided because of the possibility of breaking, which can cause a biohazard. Tubes should be plain and should not contain heparin. Alternatively, a perforating device for the stoppers of blood containers specifically designed for making blood films may be used.²

Blood Volume

The blood volume used should allow for a wedge blood film of an appropriate thickness and 2.5 to 4 cm in length. If a spinner is operated, a micropipette should be used to ensure consistent monolayers. Typically, for spun slides, 30 µL will result in a monolayer of sufficient size, but different volumes may be required for different spinners.

Position of Blood Drop

The position of the blood drop should be approximately 1 cm from the end of the slide (opposite labeling end). Alternatively, a distance of 1 cm from the label (or frosted part of the slide) can be chosen. Use the manufacturer's instructions when determining the position in the spinner.

Spreader

Ideally, the spreader should be slightly narrower than the glass slide to minimize distribution effects. In practice, a second slide is often used as the spreader, and this procedure is acceptable if the spreader slide has rounded or beveled edges, making the spread width of the slide narrower than the actual slide width.

Spreaders should be discarded after use or be cleaned thoroughly and dried before reuse. The edge of a spreader should always be smooth to ensure even thickness for the entire width of the blood film.

The presence of blood cells from a previous specimen on the spreader edge can cause significant carryover of blood cells, including malaria parasites in red blood cells and leukemic white blood cells, into the next blood film.

1. An example of an adequate slide spinner is the instrument developed by StatSpin Technologies, Norwood, MA.

2. eg, Diff-safe; Alpha Scientific, Southeastern, PA.

Blood Pick-Up by Spreader

As soon as a blood drop has been placed on the slide, the spreader should be moved slowly backwards at an angle of approximately 30° to 45° toward the blood drop. The drop should spread quickly along the spreader's edge. Once the blood is spread along the entire edge, the spreader should be moved forward immediately at a steady rate, at a fairly fast speed, and at a 45° angle until all blood has been spread into a film. The spreader should be held more upright for samples from anemic patients, creating a thicker film.

Rate of Spreading/Spinning

The faster the blood is spread on the slide, the thicker the film will be. Training and experience are required for consistent and acceptable results. It is notoriously difficult to produce acceptable wedge blood films from certain specimens such as those from newborns. For slide spinners, centrifugation speeds and times should be available from the manufacturers.

Thickness of Blood Film

The thickness of the blood film is influenced by the size of the blood drop, the patient's hemoglobin level, the angle of the spreader (the greater the angle, the thicker and shorter the blood film), and the speed of spreading.

Drying of Blood Film

Normally, air-drying without forced air circulation is sufficient. In humid conditions, forced air-drying is recommended, with proper precautions taken for aerosol formation. When forced drying is applied, it is recommended to do so in biohazard hoods equipped with high-efficiency particulate air (HEPA) filters.

Preparation Artifacts

The most common artifacts are due to poor spreading techniques, slow drying in humid conditions, insufficient or late fixation, and water containing fixing solutions. Slides that are not dry result in poorly or irregularly spread blood films, often with poor morphology.

Certain cell types can be easily damaged by blood film preparation. Specific conditions include those with large numbers of atypical lymphocytes, chronic lymphocytic leukemia (CLL), and acute leukemia. Sometimes these ruptured cells ("smudge" cells or "Gumprechtse Schollen") can be prevented by the addition of albumin to the blood sample before blood film preparation.

Slow drying causes cells to contract, whereas water in excess of 3% in the methanol fixing solution can cause gross morphological artifacts, such as decreased "crispness" of cellular appearance (especially red blood cell and nuclear) and the development of artificial vacuoles.

Degenerative changes such as cytoplasmic vacuolization in monocytes and neutrophils, nuclear lobulation or fragmentation of nucleated cells, and apoptotic changes are not so much the result of preparation but are often caused by prolonged or inadequate sample storage.

Possible Interferences

Patient conditions influencing the preparation and quality of blood films are: anemia, polycythemia, cord blood, platelet clumping, cold agglutinins, anti-red blood cell antibodies in severe hemolytic anemia, severe rouleaux formation, and blood specimens from newborns.

Blood Film Preparation Methods for Malaria Detection

It is recognized that morphological identification of malaria parasites in (thick or thin) blood films is not the most sensitive method for malaria detection. Molecular methods are much more sensitive but also much more expensive. For some time to come, therefore, the morphological identification of malaria will remain in many laboratories a major method for detection and monitoring of malaria.

Both thin and thick blood film preparations should be used for morphological detection of malaria parasites. Identification of malaria is ± 10 times more sensitive in thick preparations than in thin preparations, but the actual identification of the type of malaria parasites is more difficult in thick preparations.

For thick film preparation, a small drop of blood is placed on a glass slide and spread to approximately 4 times its original surface. After extensive drying, best done at 50°C to 60°C for 7 to 10 minutes, the slides can be stained. The cells will wash off the slide if insufficiently dried.

Two staining procedures have been described: a rapid procedure taking <30 seconds for regular blood films, and a Giemsa-based procedure for thick drop preparations, which takes approximately 30 minutes. As an alternative to thick drop preparations, a method has been described that uses blood cell lysis by saponin followed by centrifugation to remove debris [15].

Reticulocyte Preparation

Anticoagulated blood must first be incubated in a supravital dye that will stain the (mainly ribosomal) RNA in reticulocytes and cause it to aggregate into the morphologically typical dark blue network (reticulum) and granules.

Aliquots of staining solution and blood are mixed in a test tube and incubated for 15 minutes at an ambient temperature. After remixing by 10 complete inversions, at least 2 blood films are prepared on glass slides and air-dried before microscopic analysis.

Fixation/Staining

Optimal results are obtained by fixing and staining immediately after the blood film is completely air-dried. Fixation of blood films before staining is recommended, although many laboratories have a practice of staining immediately after air-drying of blood films. This procedure usually yields acceptable results. However, if slides cannot be stained immediately, fixation in methanol is necessary within 4 hours, but preferably 1 hour after air-drying; otherwise, the plasma will cause gray/blue background effects. Staining and fixing solutions must be as free of water as possible (<3%) to prevent morphological artifacts.

If manual staining procedures are used, it is recommended that slides be immersed in reagent-filled (Coplin) jars rather than covering slides with staining solution because formation of precipitate by evaporation may occur. If staining is performed under extremely hot conditions, care must be taken to prevent evaporation during staining, eg, by performing staining in a closed jar or in a closed petri dish.

Automated staining devices have their own specific staining procedures that are provided by the manufacturers. Users may modify these procedures to satisfy local requirements for cell staining.

Staining protocols vary between laboratories, and no generally accepted routine staining method or result is available. Guidelines for staining and staining results can be found in hematology and laboratory guidelines and textbooks. ICSH has published a "reference"

staining method for blood films based on purified azure B and eosin Y solutions [16].

As a general rule for judging the quality of a stained blood film, the laboratory must ensure that all cell types in a blood film can be identified reliably by the staining procedure. This rule applies equally to manual and automated methods.

Blood films are typically stained by Romanowsky dyes (consisting of a variety of thiazines and eosins). A number of methods have been described and include the following: Wright, Wright-Giemsa, May-Grünwald-Giemsa, and Leishman. Examples of commonly used methods for staining of air-dried blood films follow.

Wright Stain

Reagents

(1) Absolute methanol. (2) Staining solution (which can be purchased as a ready-made solution or as a powder from commercial sources). Typically, 0.3 g of Wright stain powder is dissolved in 100 mL absolute methanol and left in a closed container at room temperature for 24 hours. It must be filtered before use. (3) Sørensen's buffer solution at pH 6.4; KH_2PO_4 , anhydrous 6.63 g; Na_2HPO_4 , anhydrous 2.56 g; distilled water up to 1000 mL.

Method

- Step 1: Fix for at least 30 seconds in absolute methanol.
- Step 2: Remove methanol by tilting the slide.
- Step 3: Apply staining solution for 2 minutes on a horizontally positioned slide.
- Step 4: Add aliquot of the buffer solution without any of the stain running off the slide. Gently mix the buffer and stain without touching the surface of the blood film on the slide (a metallic sheen will appear on the surface of the staining solution mixture).
- Step 5: Let stand for 3 minutes.
- Step 6: Rinse the slide with (distilled) water for 30 seconds.
- Step 7: Dry the slide in a tilted position; do not blot-dry.
- Step 8: Mount a coverglass if desired.

May-Grünwald-Giemsa Stain

Reagents

(1) Absolute methanol. (2) Staining solution I: 0.3 g May-Grünwald powder in 100 mL absolute methanol; leave in closed container at room temperature for 24 hours. It must be filtered before use. Staining solution II (Giemsa stain): 1 g Giemsa stain powder is dissolved in 66 mL glycerol and heated to 56°C for 90 to 120 minutes. After addition of 66 mL absolute methanol and thorough mixing, the solution is left at room temperature in a closed container. It must be filtered before use. (3) Buffer: Sørensen's buffer solution. The pH must be at 6.8 for the May-Grünwald-Giemsa stain instead of 6.4 as in the Wright stain.

Method

- Step 1: Fix for at least 30 seconds in absolute methanol.
- Step 2: Remove methanol by tilting the slide or by simply removing from the fixing jar.
- Step 3: Apply staining solution I freshly diluted with an equal part of buffer for 5 minutes on a horizontally positioned slide or in a jar.

Step 4: Transfer slide from jar without washing (or remove staining solution by holding slide vertically) into staining solution II that has been freshly diluted with 9 parts buffer for 10 to 15 minutes.

Step 5: Transfer slide to jar with buffer for 1 rinse after removing stain.

Step 6: Wash slide with ample water.

Step 7: Transfer slide to a jar containing water for 2 to 5 minutes.

Step 8: Dry the slide in a tilted position; do not blot dry.

Step 9: Mount a coverglass if desired.

Comment

It is essential in this method that slides are not allowed to dry or evaporate between steps to prevent staining artifacts and precipitates from forming.

Malaria Stain

Regular Blood Film, Rapid Method

Reagents

Stain I: 1.3 g eosin Y in 500 mL distilled water containing 12.6 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and 6.25 g KH_2PO_4 . Let stand at room temperature for 24 hours in a closed container. Alternatively, stain I may be made by dissolving 0.8 g methylene blue and 0.5 g methylene azure (azure I—an oxidated derivative of methylene blue) in 500 mL distilled water containing 12.6 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and 6.25 g KH_2PO_4 . No evaporation step is necessary. Stain II: 1.3 g methylene blue dissolved in 500 mL distilled water containing 12.6 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and boiled to dry powder in order to “polychrome” the dye. The powder is resuspended in 500 mL distilled water, and 6.25 g KH_2PO_4 is added. Both staining solutions must be filtered before use and kept in a closed container to prevent oxidation.

Method

- Step 1: Fix slides for at least 30 seconds in absolute methanol.
- Step 2: Place 12 drops of diluted stain I on blood film (1 part stain diluted by 4 parts distilled water).
- Step 3: Immediately add 12 drops of undiluted stain II and mix with the diluted stain I already on the slide.
- Step 4: Let stand for 1 minute.
- Step 5: Drain slide and place in Sørensen's phosphate buffer, pH 6.6, for 5 seconds (for full development of staining).
- Step 6: Wash with water.
- Step 7: Dry by tilting the slide; do not blot dry.

Field's Stain

Dried but otherwise unfixed thin films and thick drop preparations can be dipped into stain I for 1 to 2 seconds followed by a rinse in Sørensen's buffer, pH 6.8, until no more stain is released from the blood film. Next the film is dipped for 1 second in stain II and rinsed immediately in the same buffer. The slide is dried in vertical position after excess water has been removed by shaking, not blotting [17].

Thick Drop Method (Giemsa)

Reagents

The staining solution is Giemsa stain, which can be purchased directly or made up by the laboratory (see “Method” under “May-Grünwald-Giemsa Stain”).

Method

- Step 1: Immerse thoroughly dried slides in a jar or, keeping the slides in a horizontal position, stain with diluted Giemsa

stain (1:10 with Sørensen's phosphate buffer at pH 6.8) for 20 to 30 minutes.

Step 2: Wash the slide with the same buffer (gently, because otherwise the blood film floats off the slide).

Step 3: Leave the slide in a tilted or upright position to dry; do not blot dry.

Comment

Because one of the goals of this technique is to lyse the red blood cells and make malaria parasites visible through several cell layers, morphology of most blood cells in thick drop preparations is poor. The prolonged immersion in the diluted staining solution makes these preparations prone to float off the slides.

Reticulocyte Stain

New methylene blue (1 g) is dissolved in 100 mL diluent (citrate-saline: 20 mL of 30 g/L sodium citrate plus 80 mL of 9 g/L sodium chloride). After the dye is dissolved, the stain must be filtered before use.

General Comments on Staining and Staining Materials

Stains and fixing materials must be kept in tightly closed containers at room temperature; freezing temperatures destroy Romanowsky stains. Blood film staining reagents should have labels that record when containers were opened; expiration dates should be on containers when applicable.

Although the raw materials are available and all stains can be made up by the laboratory, it should be noted that this is a laborious process, and in general, more consistent staining results will be obtained when ready-to-use stains are applied.

Evaluation of Stained Blood Films

Macroscopically, a properly prepared and stained blood film should be pink in its thin part and show a purple/blue tint in the thicker parts. Microscopically, the red blood cells should be pink and the nuclei of the white blood cells more purple than blue. There should be no or minimal precipitation, and staining should be uniform throughout the slide. The blood cells should be free from vacuoles (see "Fixation/Staining").

Staining Artifacts and Potential Staining Problems

Excessively pink staining can be the result of using a low pH of the buffer, insufficient staining, or excessive washing/rinsing. Staining solution that is more than 4 weeks old, especially when exposed to air, may also cause pink staining because of the formation of formic acid in methanol. *Countermeasures:* check the buffer pH, use freshly made staining solutions with methanol that has not been exposed to air, and make sure staining ingredients are correct. Sometimes the reagent lot may need to be changed. If commercial staining solutions are used and buffer pH and staining times are correct, it may be necessary to try another lot. In cold climates, make sure the staining solution did not freeze during transport.

Excessively blue staining can be the result of an alkaline pH of the buffer used, prolonged staining, or insufficient washing. Thick films will also cause cells to appear more blue. *Countermeasures:* check the buffer pH, use more diluent, and/or shorten the staining time.

Inadequately stained nuclei are often the result of insufficient staining, whereas nucleated cells in aged specimens (>8 hours after blood collection), especially when stored at ambient tem-

peratures, may show nuclear changes corresponding with apoptosis (karyorrhexis).

Precipitate may form as a result of evaporation of methanol from the staining solution or because of improper washing procedures, particularly not keeping the slide absolutely flat during the initial phase of washing. Inadequate filtration of homemade staining solutions before use, use of unclean slides, and allowing dust to settle on the blood film or slide can also cause precipitation.

Coverglass

In general, the use of coverglass (coverslipping) is not necessary. However, if blood films need to be reviewed by several individuals or need to be stored long-term, coverslipping may provide protection against mechanical damage (repeated wiping) and stain deterioration from exposure to air. A potential added advantage of covering the entire area of patient blood (stained and unstained) with coverglass is a reduction in biohazard from handling blood films.

Coverglasses should be made from the highest purity colorless glass ("water-white") and must be hydration resistant, especially in environments with high humidity. They should have uniform surface quality, be free from any irregularities, and be perfectly flat. Coverglasses typically measure 25 × 25 mm and have a thickness ranging from 0.13 to 0.17 mm (a range of other sizes is available from several distributors and manufacturers). Extra-thick coverglasses (0.17 to 0.25 mm) reduce the chances of breaking but place some restrictions on microscope use.

The requirements for cleanliness, hydration, and storage are the same as for glass slides. To prevent accumulation of dust and hydration, coverglasses should be kept in a closed container when not in use.

Automated coverslipping instruments are available for high-volume laboratories.

Mounting Media

For permanent mounting of coverglasses, synthetic resins dissolved in inorganic solvents (toluene and xylene) have largely replaced Canada balsam (Canada turpentine or balsam of fir), which is unsatisfactory because of yellowing and incomplete drying.

The mounting material should have low viscosity to ensure a thin layer of adhesive and to prevent the formation of bubbles. It should not affect optical analysis by diffraction problems or shift the color of biological stains. Antioxidants in the mounting medium can prevent discoloration of stains and of the medium itself. The material should not lose its adhesiveness over time.

The use of mounting medium as a cover for blood films without coverglass is not recommended because of the tendency of these preparations to gather dust and to be not as resistant as glass to scratching and fingerprints when stored over long periods and when viewed multiple times.

Labeling

Blood films should be labeled clearly and labels should be resistant to smudging and the effects of fixing, staining, and cleaning blood films with inorganic solvents. The label should contain at minimum patient name, date and time of collection, and sample number. It is recommended that a definitive label also contain a patient-specific identification number. Labeling by a printed label at the time of preparation of the blood film is recommended (applying the label to the back of the film may avoid staining of the label if a platen-type stainer is used).

Printed labels should be checked for possible fading over time and for sensitivity to immersion oil and solvents commonly used in the laboratory. Labeling of blood films by hand can be done using pencil, crayon (not sensitive to alcohol or other solvents), permanent marker pen, or diamond pencil.

Biohazard

General precautions apply to handling all patient specimens including blood films, whether air-dried, fixed, or stained.

Specimen Handling and Blood Film Preparation

The preparation of blood films need not be especially hazardous, but care must be taken on opening blood containers, especially when closed by a rubber stopper and not a screw cap. The removal of a rubber stopper frequently leads to droplet or microdroplet formation and must take place while covering the stopper with a wipe or similar, preferably in a biohazard hood and away from the operator's face [9].

When properly handled, microdroplet and aerosol formation does not occur in a measurable manner when tubes with screw caps are used [9]. Blood can also be safely obtained from sample tubes by using a perforating device for the stopper of a blood container specifically designed for blood film preparation.

Stained Blood Films

Intercalating dyes such as Romanowsky dyes are carcinogenic and should be handled with care, avoiding skin or mucosal contact. It is often assumed that staining blood cells with Romanowsky stains deletes infectivity, and in general, completely stained blood films are not considered a biohazard. However, methanol fixation does not provide protection against HIV or hepatitis B and is unlikely to provide protection for certain types of other infections, such as those caused by prions.

Partially Stained Blood Films

Often, only part of the blood film present on the slide is stained, and the unstained part is therefore a direct biohazard.

Coverglass Mounting Media

Some of the materials and solvents used in mounting coverglasses are potentially carcinogenic and should be handled according to the manufacturer's instructions.

Reagent Safety

The Romanowsky stains, methanol, alcohol, acetone, xylene, toluene, and coverslip glues mentioned in this article are highly flammable and should be stored in safety cabinets designated for flammable materials. When handling these reagents, their volatility and potential carcinogenicity should be taken into consideration.

Long-Term Storage

If blood films are stored for long periods, exposure to light should be avoided. Although coverslipping is recommended for long-term storage, deterioration of the films is unlikely to occur if stained slides without coverslips are kept closely packed in a dark, dry environment in closed containers or cabinets.

Disposal of Stains and Solvents

Romanowsky dyes and volatile solvents should be disposed of in accordance with local waste and disposal authority rules and regulations.

Disposal of Slides

Used slides to be discarded should be put in designated containers specifically labeled and used for discarding both biohazards and sharp objects.

Microscopy and Slide Handling

For the reasons mentioned under "Biohazard," the use of gloves is recommended while handling blood films and performing microscopic analysis. Slides with square edges are more prone to cut or puncture the skin or glove material than slides with rounded or beveled edges. Broken slides should be handled with extreme care, and unless the blood film is irreplaceable, such slides should be discarded.

Utilization of Blood Films

Blood films can be used in a number of ways. Most are used for the routine clinical quantitative and/or qualitative analysis of formed blood elements. Their use for morphological screening is probably stronger than for purely quantitative purposes now that electronic white blood cell (WBC) differential analysis has been much improved. Blood films are also used for quality control/quality assurance purposes and for the evaluation of automated instrument-based methods [18,19].

Role of Manual Differential Count as a Reference Method

For the role of blood film-based WBC differential counts as a reference method, please refer to National Committee for Clinical Laboratory Standards (NCCLS) document H20-A [20].

Considerations Apply

One consideration is that of cell distribution on wedge blood film preparations; large cells such as monocytes may be pushed to the periphery and the feathered edge of the blood film.

General Morphology Issues

Poor recognition of certain blood cell types (monocytes, nucleated red blood cells, band forms, etc.) can lead to underreporting and/or misclassification. Cell types such as hematologic progenitor cells or lymphocyte subsets are difficult or impossible to classify by morphological means alone.

Rare Event Issues

Abnormal cell types are particularly prone to underreporting. The relatively small number of cells counted, even when a total of 400 WBCs are classified, leads to significant problems in terms of accuracy and reproducibility, mainly because of Poisson error. This is particularly the case for cell types that are typically present in small numbers, such as basophils.

Because of these considerations and particularly because of cell classification issues, alternative methods such as monoclonal antibody-based flow cytometry have become more popular as a reference method, especially for rare event analysis.

Minimum Requirements for Acceptable Blood Film and Quality Control Procedures

Care should be taken to properly stain and adequately label blood films. Areas for morphological assessment should be large enough that at least 100 WBCs can be identified in samples with a WBC count within the reference interval for a specific individual.

A good-quality blood film preparation should contain a sufficiently large area for morphological evaluation.

In a wedge blood film preparation, the best area for microscopy is where red blood cells barely touch each other. Spun films typically have large monolayer areas.

Cells should not be damaged by the preparation or staining procedure or by excessive shear forces. Especially in spun films, care must be taken to ensure that none of the cell types in the monolayer are distorted by the preparative procedure. However, in certain disease conditions such as CLL, it is difficult to prevent cellular damage to the affected cells, the lymphocytes.

For repeated microscopic analysis, a coverslip should be used so that removal of immersion oil does not damage the blood films for future analysis.

Microscopy

Methods for proper microscopic analysis of blood films have been described elsewhere and are outside the scope of this article.

Quality Control

Blood films are a product of the laboratory and should therefore be subject to checks on their consistency and quality in terms of minimum requirements for acceptability. It is the responsibility of the laboratory management to put such checking procedures in place. In an entirely manual environment, sufficient training and—when necessary—remedial training should be offered to the laboratory staff to ensure that blood films of sufficient and consistent quality are being prepared. In automated and semi-automated environments, staff should be properly trained in instrument use, and records of instrument maintenance should be kept.

Proficiency Testing

The laboratory can (and is recommended to) participate in (external) proficiency testing programs, although such programs mostly test the laboratory's ability to properly stain a blood film and assess the morphological skills of the staff. In external proficiency testing programs, logistics and the aging of blood specimens usually prohibit the blood film preparation itself, especially when blood specimens are mailed out to the individual institutions.

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A number of textbooks were used as general references [21-24].

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