KCMC Biotechnology Laboratory, Microbiology	STANDARD OPERATING PROCEDURE	Effective Date 19 October 07	SOP-Number MIC.040.02
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Title: PREPARATION AND STAINING OF SMEARS FOR BLOOD PARASITES

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Annual Review	
By	**Date**

Title: PREPARATION AND STAINING OF SMEARS FOR BLOOD PARASITES

Document History:

Version Number	Reason for Changes	Date
MIC.040.02	Addition of Giemsa stain preparation and staining using commercial stock stain.	19 October 2007

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Name	Date

Title: PREPARATION AND STAINING OF SMEARS FOR BLOOD PARASITES

PURPOSE
Procedures for preparation and staining of thick and thin blood smears for examination for blood parasites.

PRINCIPLE
Whole blood is collected in a vacutainer tube containing EDTA anticoagulant. A large drop of blood is spread in a small circle on a clean, grease-free microscope slide and allowed to air dry (thick smear). A second slide is prepared with a small drop of blood and spread thinly over the slide surface (thin smear). The thin smear will be fixed with methanol prior to staining to preserve the red cell morphology. The thick smear is not fixed and the RBCs lyse during the staining process. Both slides are stained with Giemsa and examined for malaria and other blood parasites. The thick blood film is about 30 times more sensitive than the thin film (detecting about 20 parasites/μl**)** and is most suitable for the rapid detection of blood parasites, particularly when they are few. The thin film with intact RBCs is examined for speciation of malarial parasites if this is not apparent on the thick smear.

SCOPE
This Standard Operating procedure applies to all personnel who prepare and stain slides for blood parasites and are trained and competent in performing these procedures.

STANDARD PRECAUTIONS
Standard precautions should be observed when obtaining and handling patient samples, preparing and handling unstained slides. Wear gloves to prevent exposure to bloodborne pathogens.

SPECIMEN COLLECTION AND HANDLING

Specimen Type: Whole blood collected in EDTA vacutainer tube. Refer top MIC.012 SPECIMEN COLLECTION ISAAC STUDIES for blood collection procedure.

Specimen Handling
Refer to SOP MIC.011 SPECIMEN RECEIPT AND HANDLING for minimal volumes, rejection criteria, pre and post analysis handling and storage.

SMEAR PREPARATION

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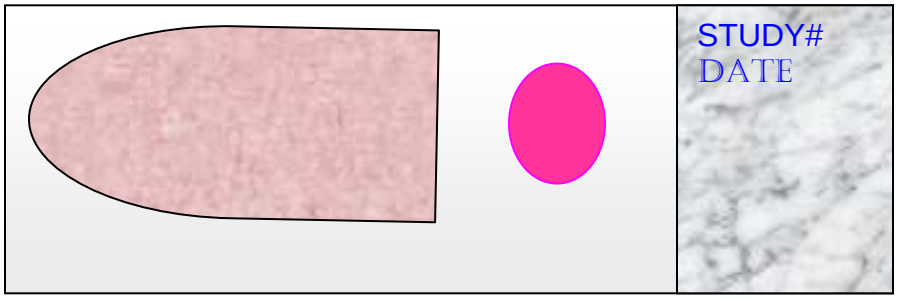
Materials:

Glass slide cleaned with 70% alcohol to remove grease and film.
Second slide for spreading thin smear ("spreader slide")
Pasteur pipette
Pencil
Slide box

PROCEDURE

1. Label slide with the following:
   Study ID#
   Date of collection
2. Clean the surface of each slide with 70% alcohol to remove dust and grease.
3. Mix blood sample by gently inverting 5 times.
4. THICK SMEAR: Using a pasteur pipette dispense one large drop (6 ul) of blood near the frosted end of the slide and spread to a circle with approximately a 1.5 cm diameter.
5. THIN SMEAR: Using a Pasteur pipette, place a small drop (2 ul) of blood below the area of the thick smear.
6. Hold the "spreader slide" at a 35-40^{o} angle up to the drop, and allow the drop to spread along the contact line of the 2 slides.
7. Quickly push the spreader slide toward the unfrosted end of the slide.
8. Make sure the spread film has good, feathered edge. Repeat if necessary.
9. Allow the smear to air-dry with the slide in a horizontal position.
10. Place slides in covered slide box.

Diagram of smear appearance:



Thin Smear **Thick smear**

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PREPARATION OF GIEMSA STAIN FROM COMMERCIAL STOCK SOLUTION

Materials:

ACCUSTAIN Giemsa Stain (Sigma-Aldrich #GS500)
17x100mm test tube with graduations for diluting.

Procedure (prepare fresh each day):

1. Dilute stock Giemsa stain 1:20 with distilled water (add 2 ml stain to 8 ml water)
2. Mix thoroughly by inverting tube.
3. Discard stain at the end of the day.

PREPARATION OF GIEMSA STAIN STOCK SOLUTION FROM POWDER

Materials:

Giemsa stain powder
Glycerol
Methanol (absolute)
Measuring cylinder
Funnel & filter paper
Balance for weighing ingredients
Mixer
Brown bottle

Procedure (For 1 liter of stock stain solution):

1. Measure 500 ml of glycerol in a graduated cylinder and pour into dark bottle.
2. Add 500 ml absolute methyl alcohol.
3. Add 7.6 grams Giemsa powder.
4. Allow the contents to mix overnight using a mixing machine.
5. Once thoroughly mixed, filter mixture using the filter paper and funnel.
6. Store the filtered solution in brown bottle in a cupboard to avoid exposure to direct sunlight.
7. Cleary label the bottle with the date of preparation, date of expiration 6 months from date of preparation and the initials of the preparer.

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PEPARATION OF BUFFER SOLUTION pH 7. 2

Materials:

Sodium phosphate, monobasic dehydrate (NaH_2PO_4)
Potassium phosphate monobasic (KH_2PO_4)
Distilled water
Measuring cylinder
pH meter
Glass bottle
Weighing balance

Preparation:

1. Weigh 1.09 g $NaH_2 PO_4$
2. Weigh 0.7 g $KH_2 PO_4$.
3. Dissolve both in 1000ml distilled water
4. Measure pH. using pH meter.
5. If necessary, add small quantities of 0.1N HCl or 0.1 N NaOH to bring the PH of the buffer to pH 7.2
6. Put solution in glass bottle.
7. Autoclave at 121^{o} C for 15 min.
8. Label with date of preparation, expiration date 1 year from date of preparation and initials of preparer.
9. Store at room temperature away from sunlight.

PREPARATION OF WORKING STAIN SOLUTION

Prepare fresh each day of use.

Materials:

Giemsa stock solution
Buffer solution. pH. 7.2
Measuring cylinder

Preparation:

1. Prepare a 3% solution of the Stock Giemsa stain in phosphate buffer (e.g. For 100 ml of working stain solution add 3 ml of stock Giemsa stain to 97 ml of buffer.)
2. Discard unused solution at the end of each day.

GIEMSA STAIN PROCEDURE – STAIN PREPARED FROM COMMERCIAL STOCK

1. Fix THIN SMEAR with methanol for 5 minutes by immersing in Copelin jar **up to, but not including the THICK SMEAR.**
2. Allow smear to air dry.
3. Place smear on a staining rack and flood slide with diluted Giemsa stain solution or fill Copelin jar and add slides.
4. Stain for 60 min.
5. Rinse smear gently with water to remove stain.
6. Allow to air dry.

GIEMSA STAIN PROCEDURE – STAIN PREPARED FROM POWDER

1. Flood THIN SMEAR with methanol for 1 minute or immerse in Copelin jar for 1 minute up to, but not including THICK SMEAR. ***DO NOT FIX THICK SMEAR WITH METHANOL.***
2. Allow to air dry.
3. Place smear on a staining rack and flood slide with working stain solution or fill Copelin jar with the working stain solution and add slides.
4. Stain for 30-45 min.
5. Rinse smear gently with buffer to remove stain or dip in Copelin jar containing buffer 3-4 times.
6. Allow to air dry.

QUALITY CONTROL

Frequency: Each new batch of stain prepared from powder or each lot of commercial stock stain.

Control: Thin smears prepared from blood from patient with high parasitemia (one parasite per 2-3 hpf), fixed in methanol and stored at -80^{o} C.

Acceptable Results:

1. Background should be clear and free from debris.
2. Nuclei of WBCs stain deep purple, cytoplasm pale purplish-blue.
3. RBCs stain pale pink.
4. Malaria parasite – blue rings, red chromatin dot.

Corrective Action for Unacceptable Results:

1. Restain new slide under supervision and reexamine.
2. If still unsatisfactory, prepare fresh stain and repeat QC.

Documentation:

1. Record QC results on GIEMSA STAIN QC SHEET.
2. Document all corrective actions for unacceptable results on QC Deviation form and submit to supervisor for review.
3. Review QC results monthly.

REFERENCES

MalariaGEN: Guidelines for Diagnostic Procedures for Blood Samples, Centers for Disease Control, Atlanta, GA.
Diagnostic Procedures for Blood Specimens for, Centers for Disease Control, Atlanta, GA, July 2003.

APPENDIX A - Giemsa Stain QC sheet