
بسم الله الرحمن الرحيم

Prepare & Staining of Blood Smear

by: Alizadeh Sh Ph.D

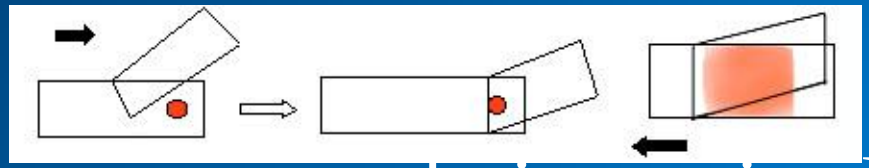
Making blood film

- Blood film can be prepared from fresh blood without anticoagulant or from EDTA anticoagulanted blood.
- blood film should be made on clean glass .
- Clear without any dust

Blood smears

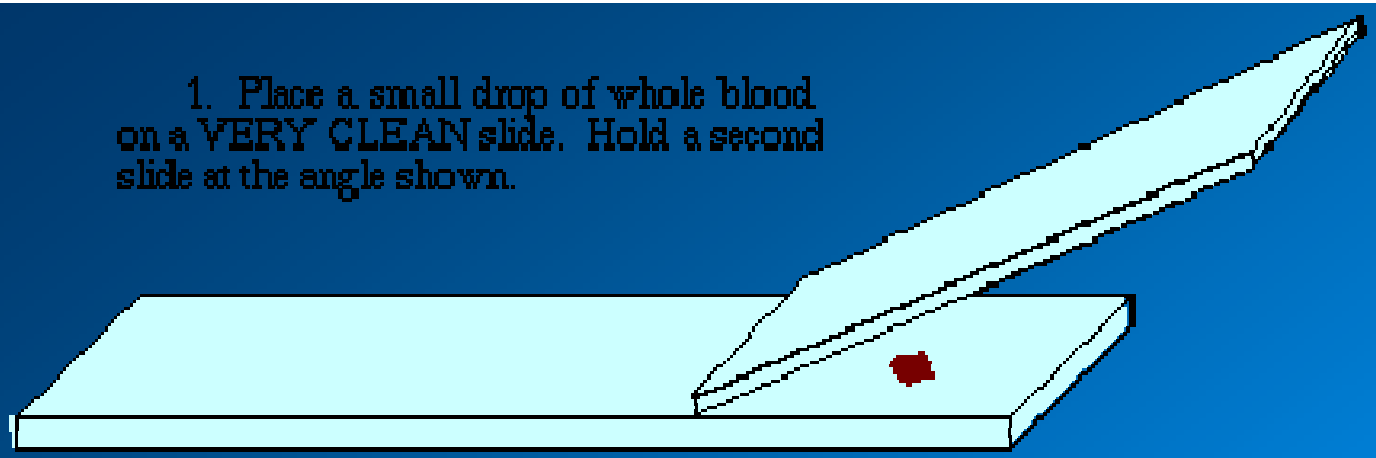
- *Coverglass method*
- *Wedge method*
- *Spun*
- *Wet smear*
- *Thick smear*
- *Buffy coat smear*

Wedge method

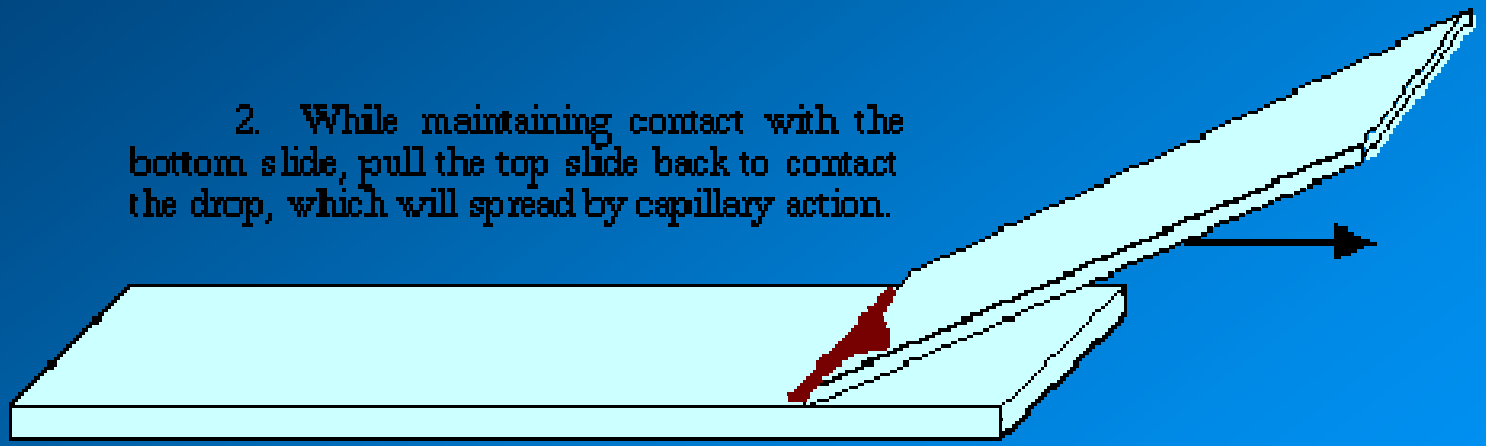


- The most commonly in routine lab
- Method
- Thickness or thinness regulated by
- Amount of blood
- Speed of spreader
- Angle

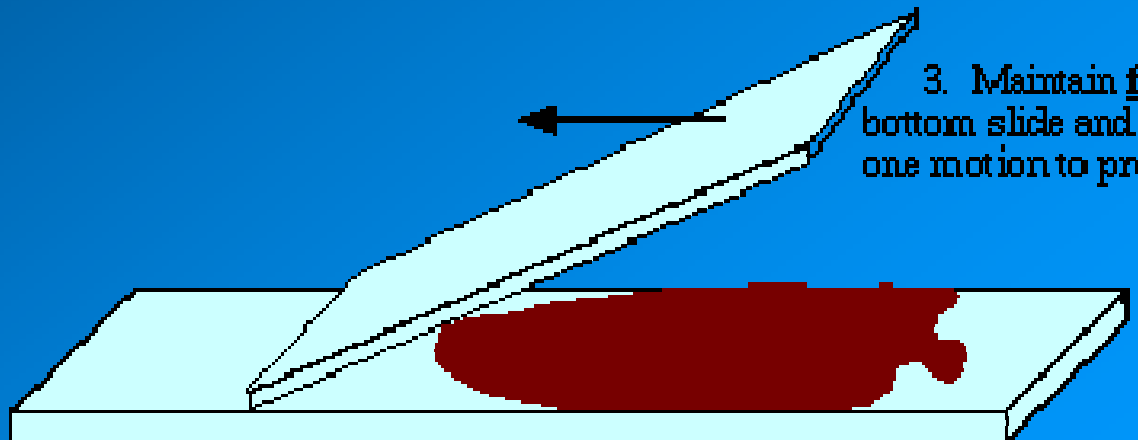
1. Place a small drop of whole blood on a VERY CLEAN slide. Hold a second slide at the angle shown.



2. While maintaining contact with the bottom slide, pull the top slide back to contact the drop, which will spread by capillary action.



3. Maintain <u>firm</u> contact with the bottom slide and push the top slide in one motion to produce the smear.



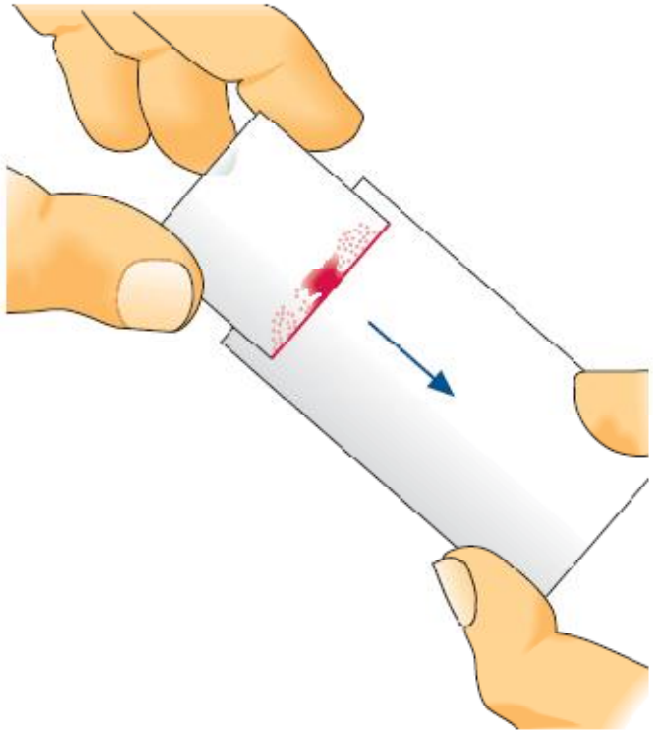


Fig. **4** Preparation of a blood smear

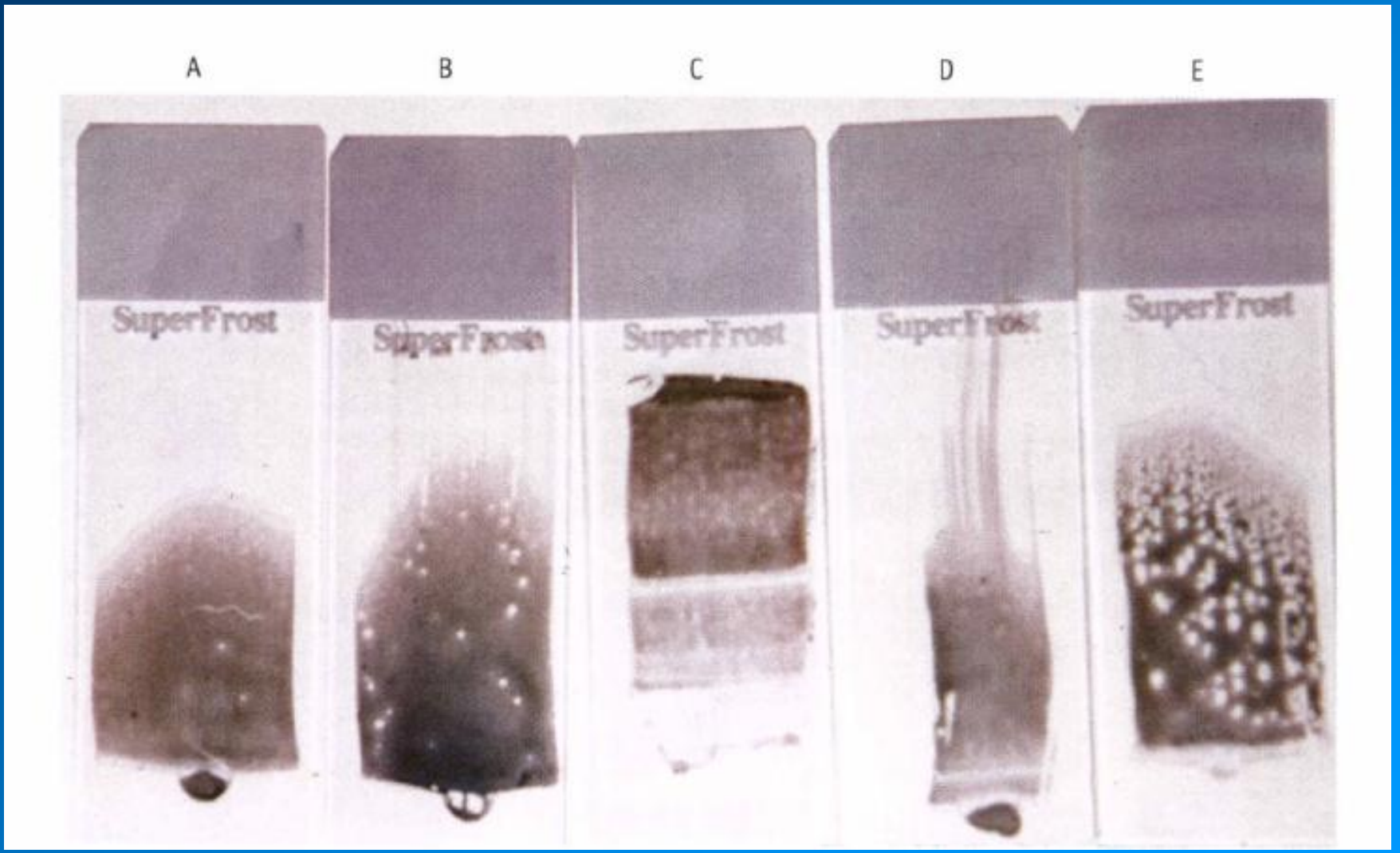
Optimal blood smear characteristic

- minimum 2.5 cm in length terminating at least 1 cm from the end of the slide
- Gradual Transition in thickness from thick to thin area ending in a Square or straight edge
- No streaks , waves , or troughs

Optimal size of Smear (wedge)

- Length: 2.1-3.3 cm
- Width: 2-2.5cm
- Thick area 1-1.5cm
- Detection of Hb C crystal
- Detection of Basophilic stippling
- Detection of Microfilerias
- Determination of Malaria subtypes

- Proper area 1-1.5 cm
- Terminal thin area 0.1-0.3 cm


A
B
C
D
E
SuperFrost
SuperFrost
SuperFrost
SuperFrost
SuperFrost

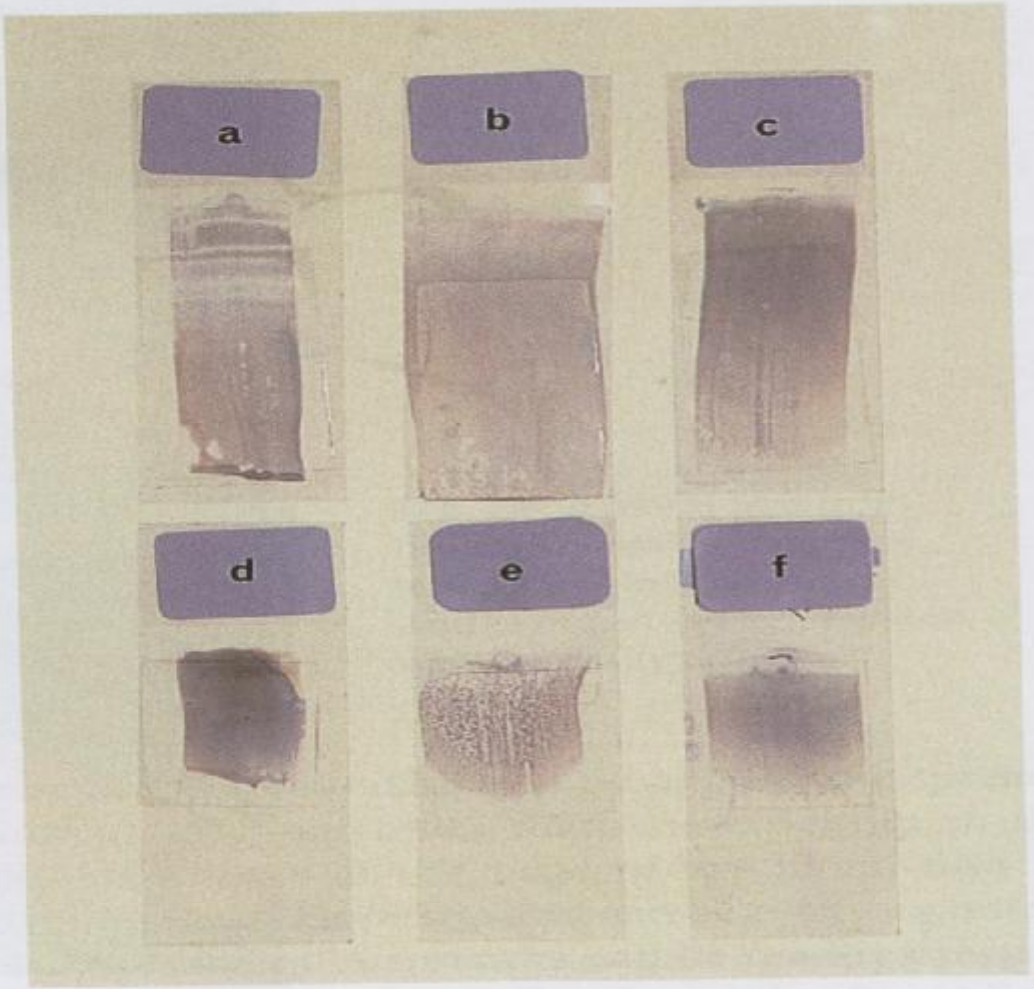


Fig. 1.11 Unsatisfactory and satisfactory blood films: (a) uneven pressure has produced ridges; (b) too broad and too long—the edges and the tail of the film cannot be examined adequately; (c) too long and streaked by an uneven spreader; (d) too thick and short due to the wrong angle or speed of spreading; (e) even distribution of blood cells has been interrupted because the slide was greasy; and (f) satisfactory.

جدول ۴. خطاهای شایع در تهیه گسترش

نوع خطا	علت
گسترش پله پله، برآمدگی و فرورفتگی	لرزش دست در هنگام تهیه گسترش
مشخص نبودن ناحیه انتهایی	قطره خون بسیار بزرگ
صاف بودن انتهای گسترش بصورت خط ضخیم	زودبرداشتن لام سنجش کننده
انتهای دندانه دار	ناصاف بودن لام گسترش دهنده
وجود خطوط طولانی ناهمگون در گسترش	وجود لخته خون در لام
وجود حفره در گسترش	چرب بودن لام گسترش دهنده – شیلومیکرونمی – نسبت بالای ضد انعقاد به خون

Sources of error in preparation of a blood smear

problem	*Resolution*
Presence of crenated erythrocyte	Dry smear quickly and thoroughly
Thin smear due to anemia	Increased spreader slide angle and increased push speed
Thick smear due to polycythemia	Decrease spreader slide angle and decrease push speed
Presence of agglutinated erythrocytes associated with cold agglutinin disease	Warm blood in 37°C for 15 min prior to preparing smear
Increased viscosity associated with multiple myeloma	Decrease spreader slide angle and decrease push speed

error in preparation of a blood smear from excess press

- *Crescent cell*
- *Selenoid body*
- *Crushed cell*
- *Broken cells*
- *Smudge cell*
- *Basket cell*

Spinner

- Blood films that combine the advantages of easy handling of the wedge slide & uniform distribution of cells of the coverglass reparation
- Method
- Advantages: minimal exposure to biohazards , increased optimal counting area

Wet smear

- Detection of parasits: Burelia, Tripanosoms, Micrfilerias
- Detection of IntraRBC Parasits: Malaria, Babesia
- Detection of IntraRBC Bacteria: Bartonella Baciliformis (type)
- Detection of RBC dismprphology: Crenated RBC
- Detection of RBC inclusion bodies (no type)

fixation

Fixation →Methanol<4%water, with 1 hour

Delay ： Adherence of Pro. To slide → Blue background

- Alcohol change or CuSo4
- Artifact hypochromia

Romanowsky stain

- Romanowsky stain→halogenated fluorescein dye, usually eosin B or Y and methylene blue and/or its products of oxidation (azure B),)

  Eosin:Acidic Dye bind to Basic groups (Hb,Granules) → reddish or orange color

  Azure B: Dye bind to nucleic acid & nucleoproteins →Blue-violet color

Romanowsky stain

- Wright
- Wright – Giemsa
- Lishman
- May- grunwald - Giemsa
- jenner

Preparation of Solutions of Romanowsky Dyes

May-Grünwald Stain

- Weigh out 0.3 g of the powdered dye and transfer to a conical flask of 200–250 ml capacity. Add 100 ml of methanol and warm the mixture to 50°C. Allow the flask to cool to *c* 20°C and shake several times during the day. After letting it stand for 24 hours, filter the solution. It is then ready for use, no “ripening” being required.

Jenner's Stain

- Prepare a 5 g/l solution in methanol in exactly the same way as described earlier for the May-Grünwald stain.

Giemsa's Stain

- Weigh 1 g of the powdered dye and transfer to a conical flask of 200–250 ml capacity. Add 100 ml of methanol and warm the mixture to 50°C; keep at this temperature for 15 min with occasional shaking, then filter the solution. It is then ready for use, but it will improve on standing for a few hours.

Azure B-Eosin Y Stock Solution

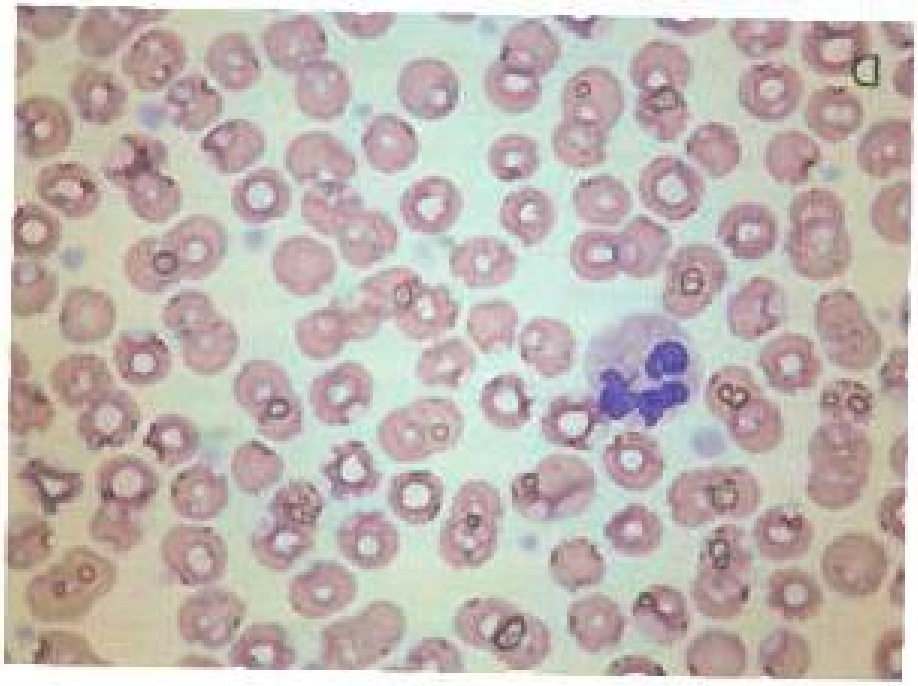
- The stock solution includes azure B, tetrafluoroborate, or thiocyanate (Colour index 52010), >80% pure, and Eosin Y (Colour index 45380), >80% pure.
- Dissolve 0.6 g of azure B in 60 ml dimethyl sulphoxide (DMSO) and 0.2 g of eosin Y in 50 ml DMSO; preheat the DMSO at 37°C before adding the dyes. Stand at 37°C, shaking vigorously for 30 sec at 5-min intervals until both dyes are completely dissolved. Add the eosin Y solution to the azure B solution and stir well. This stock solution should remain stable for several months if kept at room temperature in the dark. DMSO will crystallize below 18°C; if necessary, allow it to redissolve before use.

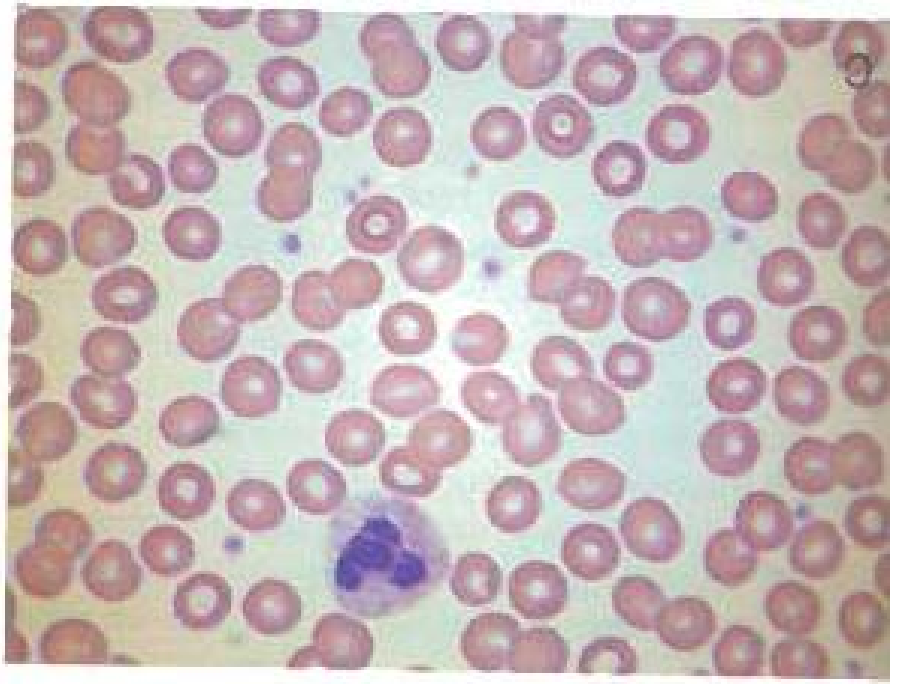
Leishman's Stain

- Weigh out 0.2 g of the powdered dye, and transfer it to a conical flask of 200–250 ml capacity. Add 100 ml of methanol and warm the mixture to 50°C for 15 min, occasionally shaking it. Allow the flask to cool and filter. It is then ready for use, but it will improve on standing.

Buffered Water

- Make up 50 ml of 66 mmol/l Sörensen's phosphate buffer of the required pH to 1 litre with water at a pH of 6.8 . An alternative buffer may be prepared from buffer tablets, which are available commercially. Solutions of the required pH are obtained by dissolving the tablets in water.


D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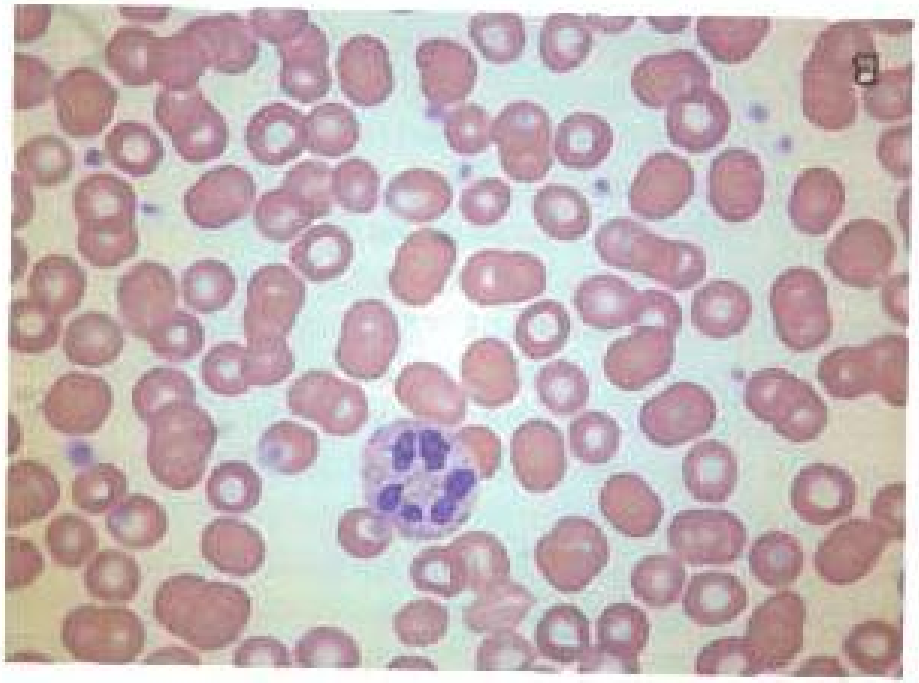
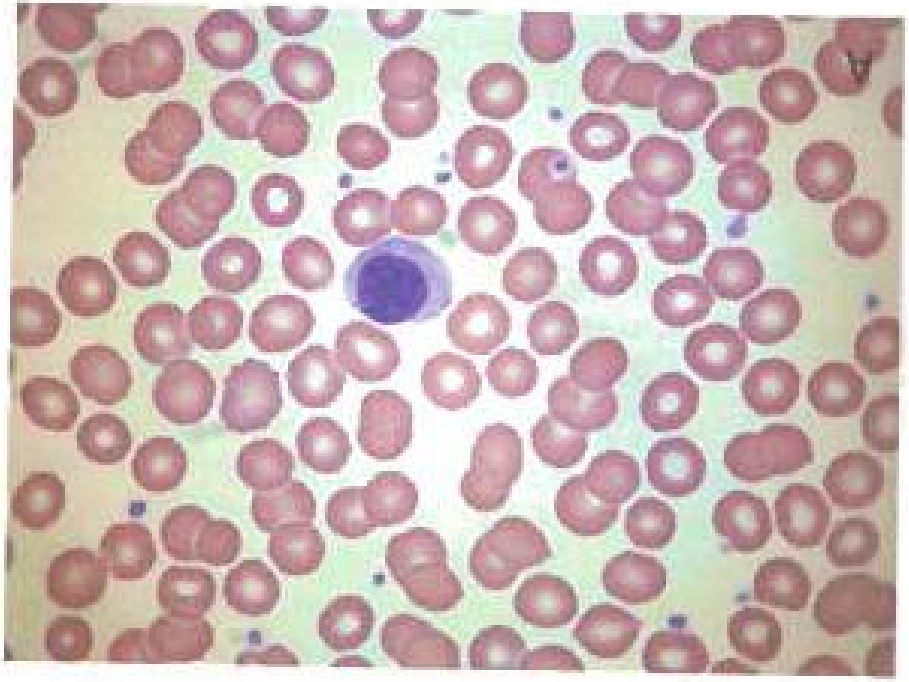

B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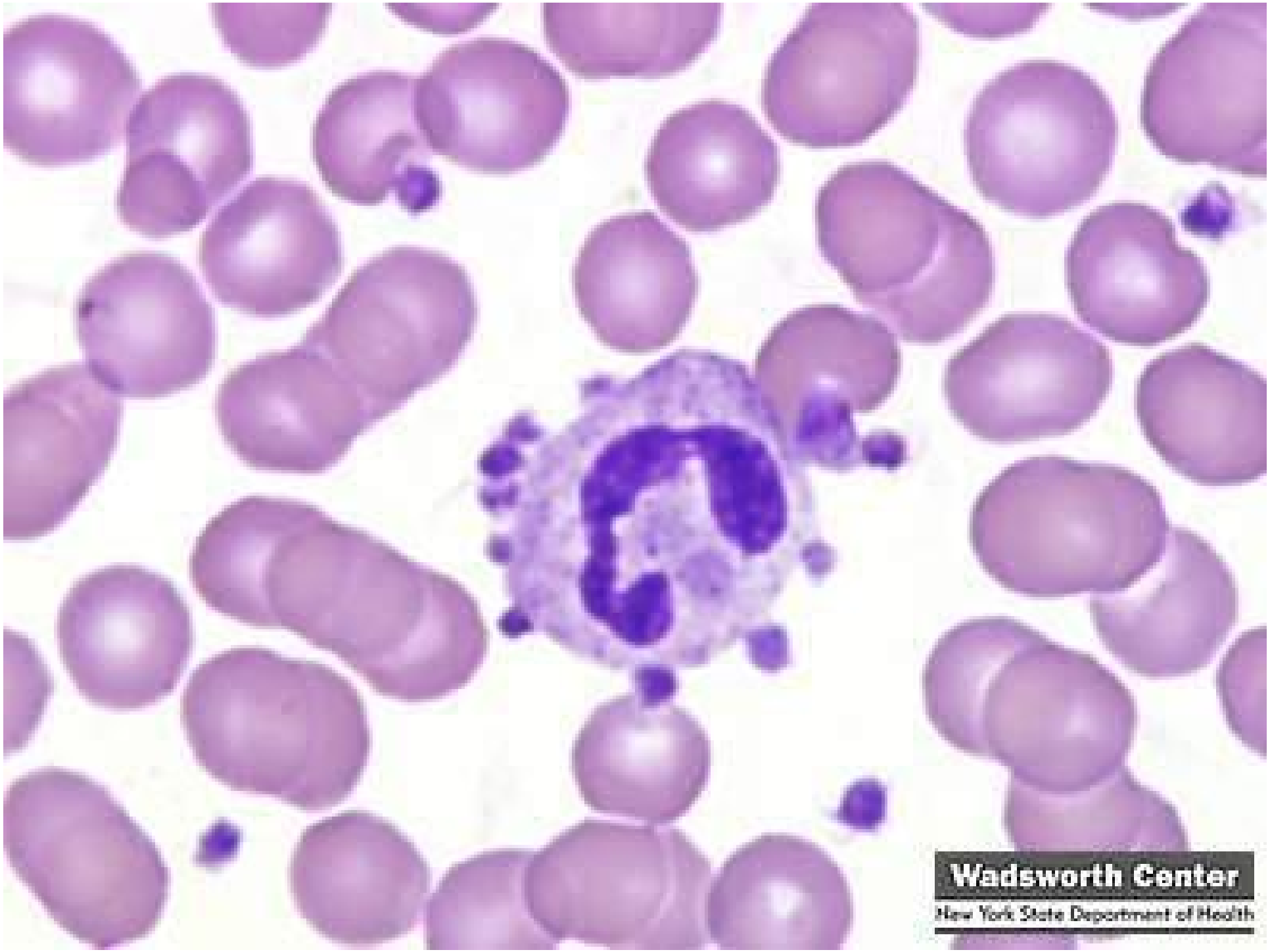

Wadsworth Center
New York State Department of Health

Reference method

- Pure Azure B (260mg/100ml methanol)
- Pure eosin y (130 mg/100ml methanol)
- 1 part Azure B + 1 part eosin y +10 part sorensens phosphate buffer 66mmol/l pH= 6.8
- 10 min
- washing

Characteristic of a properly stained blood smear

Type of evaluation	*Characteristic*
Macroscopic	Smear is pinkish purple in color
Microscopic	Blood cells are evenly distributed Areas between cells are clear Erythrocytes are orange red Neutrophilic granules are lilac Eosinophilic granules are red orange Lymphocytes cytoplasm is blue Leukocytes nuclei are purple Precipitated stain is minimal or absent

problem	***Potential causes***
Excessively blue or dark stain	Prolonged staining Inadequate washing Too high an alkalinity of stain and / or buffer Thick blood smear
Excessively pink or light stain	Insufficient staining Prolonged washing Too high an acidity of stain and / or buffer
Presence of precipitate	Unclean slides Drying during staining process Inadequate filtration of stain

problem	***Potential causes***
Pale staining	Old staining solution overused staining solution Impure dyes High ambient temperature
Blue Background	Prolonged storage before fixation Blood collection into heparins anticoagulant

problem	***Potential causes***
Too blue Nuclei	Eosin concentration too low
Neutrophil granules not stained	Insufficient azure B
Neutrophil granules Dark blue/black (pseudo toxic)	Excess azure B

Quality Control

رنگ پس از تهیه از نظر آلودگی قارچی و میکروبی وهر گونه رسوب و پارتیکل وهمچنین نحوه رنگ گرفتن سلول های خونی بررسی می گردد.

رنگ آمیزی گسترش های خونی روتین نیز هفته ای یک بار توسط مسئول فنی داخلی از نظر موارد فوق بررسی می گردد که بصورت مکتوب ومستند باید در آزمای‌شگاه قرار گیرد.

Nuclei	
Chromatin	Purple
Nucleoli	Light blue
Cytoplasm	
Erythroblast	Dark blue
Erythrocyte	Dark pink
Reticulocyte	Grey–blue
Lymphocyte	Blue
Metamyelocyte	Pink
Monocyte	Grey–blue
Myelocyte	Pink
Neutrophil	Pink/orange
Promyelocyte	Blue
Basophil	Blue
Granules	
Promyelocyte (primary granules)	Red or purple
Basophil	Purple black
Eosinophil	Red–orange
Neutrophil	Purple
Toxic granules	Dark blue
Platelet	Purple
Other inclusions	
Auer body	Purple
Cabot ring	Purple
Howell-Jolly body	Purple
Döhle body	Light blue

Smear inspection

RBC Morphology Grading

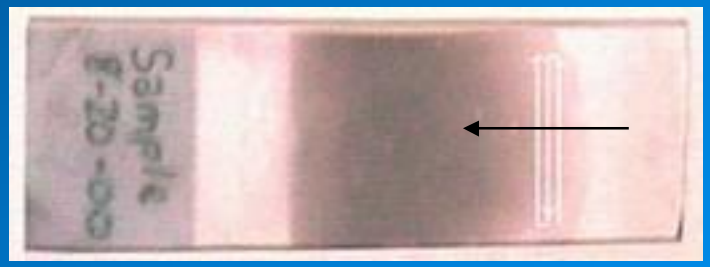
- The MCV parameter must be compatible with the blood film before the microcytosis and macrocytosis are graded.

Grade	+	++	+++
Interpretation	Slight/ A few number	Moderate/ Moderate number	marked/ numerous
Microcytosis	MCV : 70 - 79	MCV : 60 - 69	MCV <60
Macrocytosis	MCV : 100 - 115	MCV : 115 - 125	MCV >125
Hypochromasia	MCH : 23 - 26	MCH : 21 - 23	MCH <20

Grade	+	++	+++
Interpretation	Slight/ A few number	Moderate/ Moderate number	marked/ numerous
Polychromasia	3 - 5%	5 - 25%	>25%
Spherocytosis	1 - 5%	5 - 25%	>25%
Schistocytosis	up to 2%	2 - 25%	>25%
Target cells (codocytes)	up to 3%	3 - 25%	>25%
Tear drops	up to 2%	2 - 25%	>25%
Burr cells	1 - 3%	3 - 10%	>10%
Sickle cell(drepanocytes)	3 - 5%	5 - 25%	>25%
Elliptocytosis	1 - 5%	5 - 25%	>25%
Basophilic stipplings	up to 2%	2 - 25%	>25%
Howell Jolly bodies	up to 1%	2 - 3 %	>3%
Anisocytosis	RDW: 16-18	RDW : 18-22	RDW>22

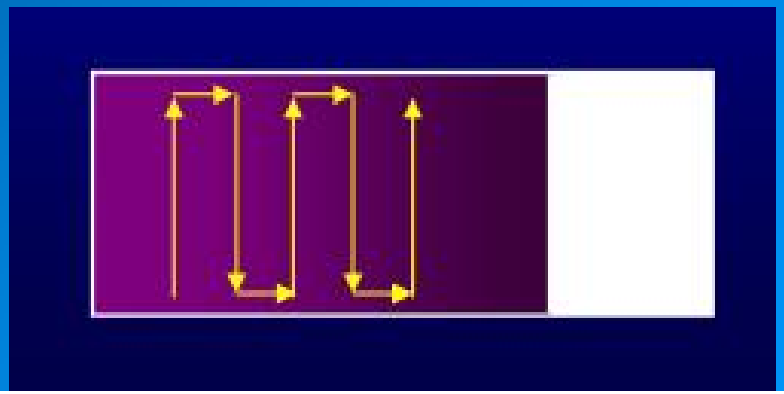
WBC Diff

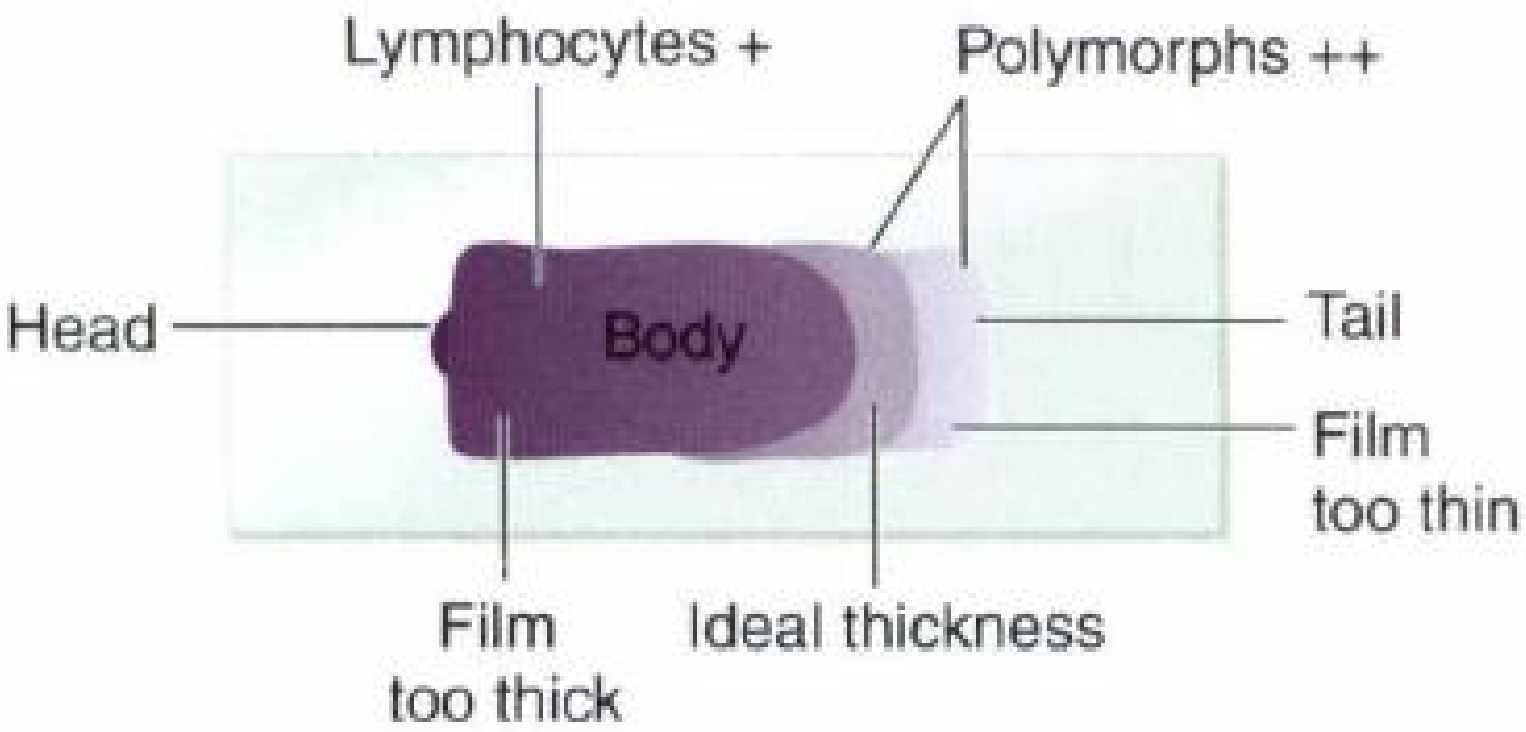
- Longitudinal method

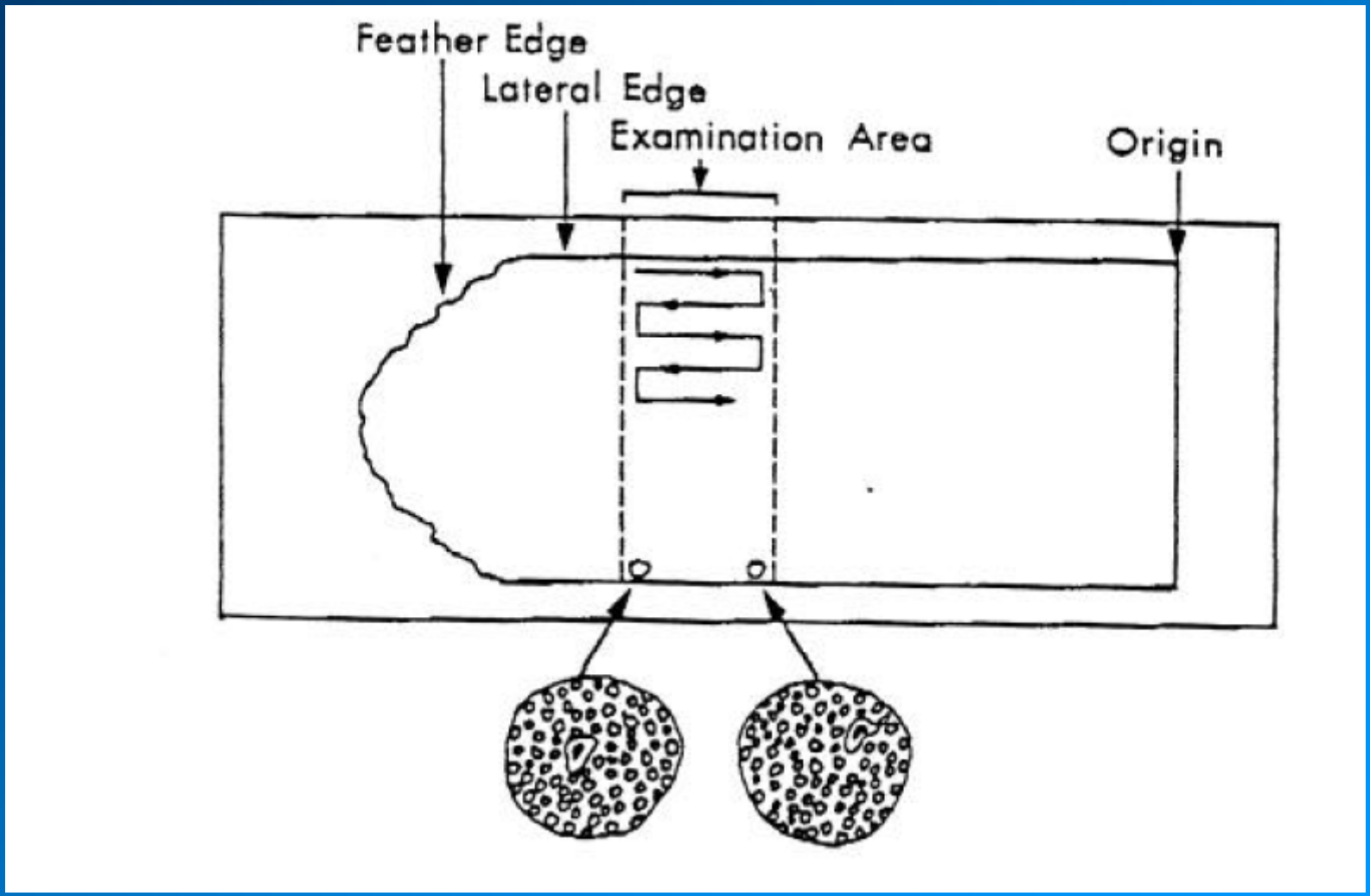


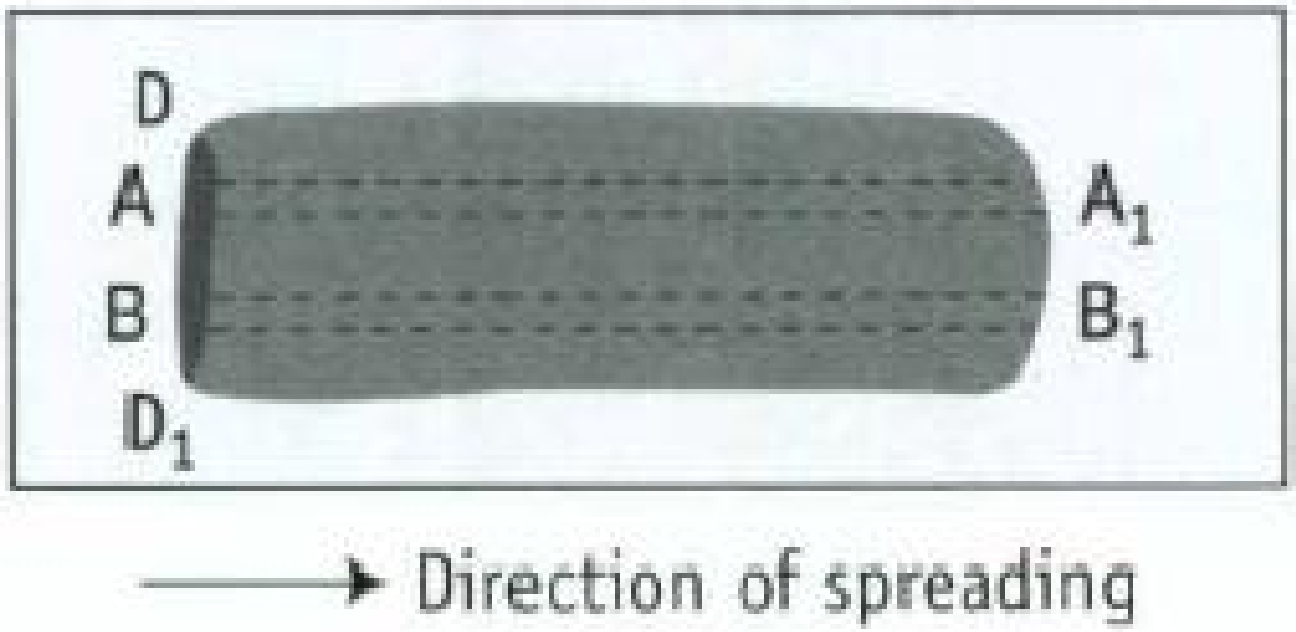
- Width method

- Battlement method (zigzag method)

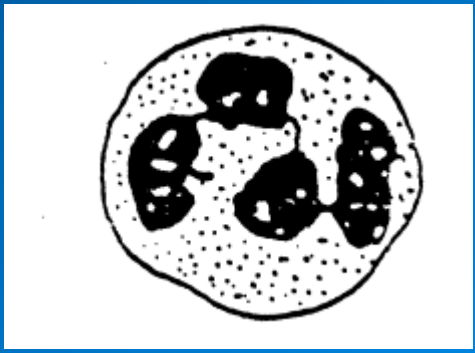



Lymphocytes +
Polymorphs ++
Head
Body
Tail
Film
too thin
Film
too thick
Ideal thickness


Feather Edge
Lateral Edge
Examination Area
Origin


D
A
B
D1
A1
B1
Direction of spreading

Neutrophil, Segmented Form (Polymorphonuclear Leukocyte)



- (1) **Round**, and approximately 15 Fm in diameter.
- (2) Cytoplasm stains light pink with specific or secondary granules of fairly uniform size.
- (3) Specific granules are evenly distributed, variable in number, and pink to lavender in color.
- (4) The nucleus is lobulated; the lobes are connected by thread-like filaments.
- (5) The elongated nucleus may be folded over; the lobes may be touching each other or may be superimposed, and various nuclear appendages may be seen.
- (6) Nuclear chromatin forms dark, densely stained blocks separated by a network of lighter purple bands.
- (7) Occasionally, large, dark, coarse cytoplasmic granules are seen in patients with infections or other serious illnesses; these are called toxic granules.

Neutrophil, Band or Stab Form

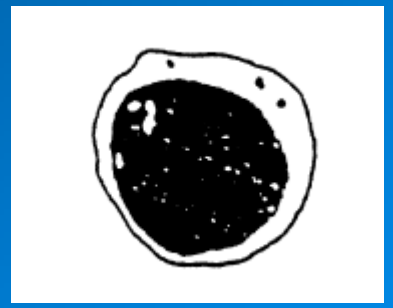
- (1) Similar to segmented forms in size and in cytoplasmic characteristics; different from segmented neutrophils in that the connection between the end of the beginning lobe formation of the nucleus is **band-like rather than a filament**. The connecting band, or isthmus, is wide enough to reveal two distinct margins surrounding nuclear material.

- (2) Characteristically, the nucleus is elongated with rounded ends and with an area of pyknosis at each pole.

- (3) The nucleus is curved or sausage-shaped, and the sides are parallel over an appreciable distance.

- (4) Less typical band forms have **multiple lobes interconnected by wider bands** instead of filaments.
- ***If the examiner is not sure whether a neutrophil is a band form or a segmented form, it is arbitrarily classified as a segmented neutrophil.***

Lymphocytes, Normal Forms



- (1) 7-15Fm in diameter, but generally, 7 to 12 Fm.
- (2) Round, or at times slightly indented.
- (3) The basophilic cytoplasm ranges from abundant to sparse and stains pale or bright blue; sometimes the stained cytoplasm appears uneven or bubbly.
- (4) The cytoplasm may contain relatively large azurophilic granules.
- (5) The nuclei vary in size, shape, and chromatin pattern.
- (6) Although usually round, the nuclei may be kidney shaped. Notching or clefts may be seen, or they may be lobulated or folded.
- (7) The chromatin is arranged in densely staining compact blocks separated by lighter tones without sharp demarcation. Sometimes a fine chromatin pattern is seen, with evident nucleoli; at other times the chromatin appears filmy, coarsely granular, or ropy.

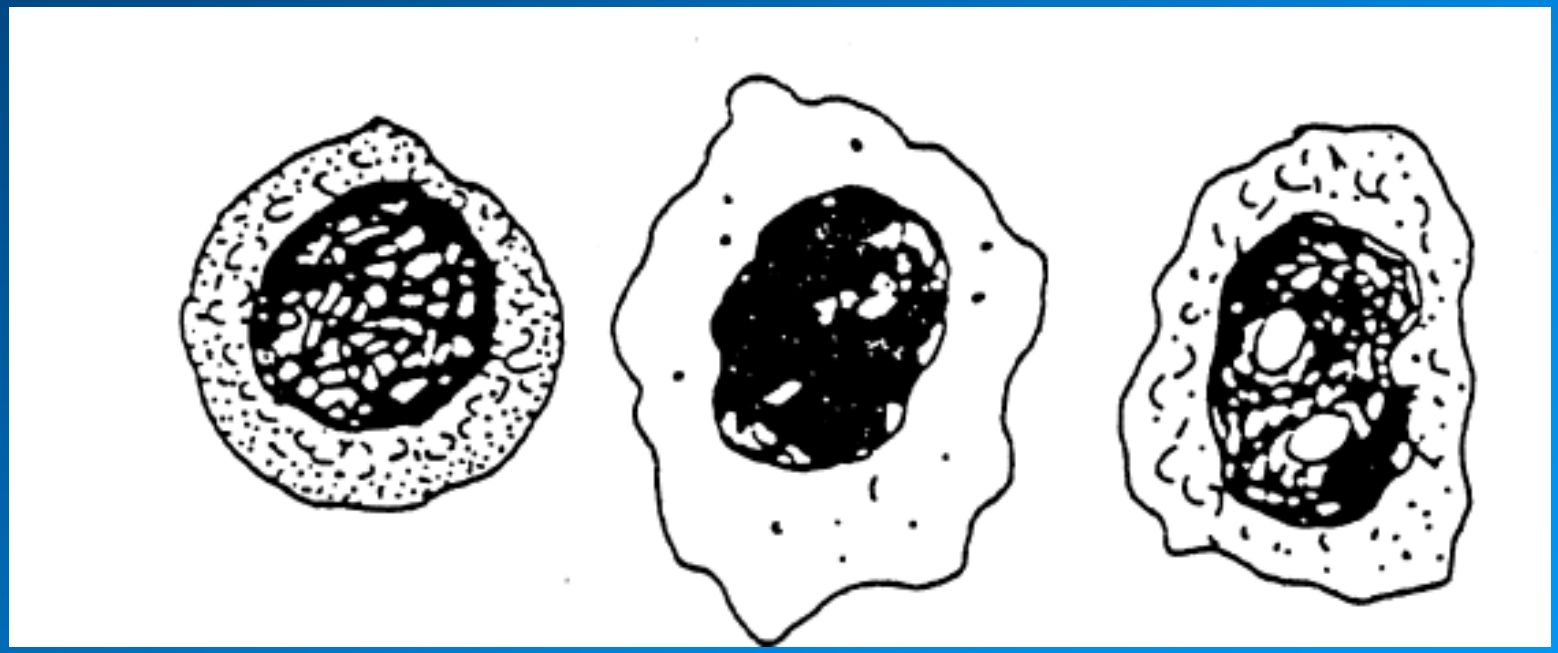
Lymphocytes, Variant Forms

- (1) These cells can be **normal physiologic variants** or **abnormal forms**. These cells are large and quite variable in appearance. The terms ***"atypical, reactive, Downey cell, virocyte"*** etc., have been used to identify these cells. Because of confusion about the relationship of these cells to either benign or malignant processes, the subcommittee chose the new term—***lymphocytes, variant forms.***

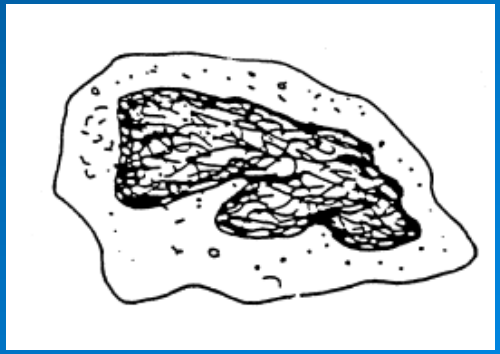
- (2) The cytoplasm may be abundant, often appear foamy, or even frankly vacuolated.

- (3) Increased cytoplasmic basophilia may be noted, especially at points of contact with adjacent cells; usually, the cytoplasmic staining ranges from blue-gray to light blue.

- (4) The nuclear chromatin may be dense, lumpy, or "blocked" with clearer areas of parachromatin; nucleoli may be visible. **A normal differential count usually includes up to 6% of variant forms.** Transitional forms between normal and variant lymphocytes are also found. In children in apparently good health, more immature-appearing lymphocytes with clear nucleoli are sometimes found.



Monocytes



- (1) Usually larger than neutrophils, ranging from 12 to 22 Fm in diameter.

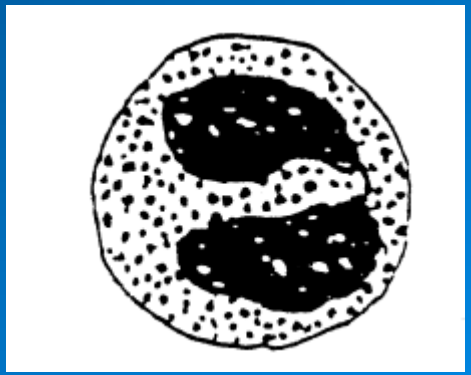
- (2) Although the majority are round with smooth margins, some monocytes may have one or more large, or multiple smaller, blunt cytoplasmic protrusions.

- (3) The cytoplasm stains gray-blue and contains numerous small, poorly defined granules causing a "ground glass"-like appearance. Sometimes numerous, dust-like and/or discrete azurophilic granules are seen. Vacuoles are common and phagocytosed particles are sometimes seen.

- (4) The nuclei are quite variable in shape and may be round, oval, indented, deeply lobulated, or even segmented. In most cases the nucleus shows some degree of folding or brain-like convolutions.

- (5) The chromatin stains light purple and lacy, although it may be coarse at times.

Eosinophils



- (1) Eosinophilic granulocytes are slightly larger than neutrophils, usually 12 to 16 Fm in diameter.
- (2) The cytoplasm contains many large spherical refractile granules, uniform in size, which stain from bluish-red to bright orange-red. The granules are usually evenly distributed, fill the cytoplasm but rarely overlay the nucleus.
- (3) The nucleus is usually segmented into two or three lobes, and occasionally more.

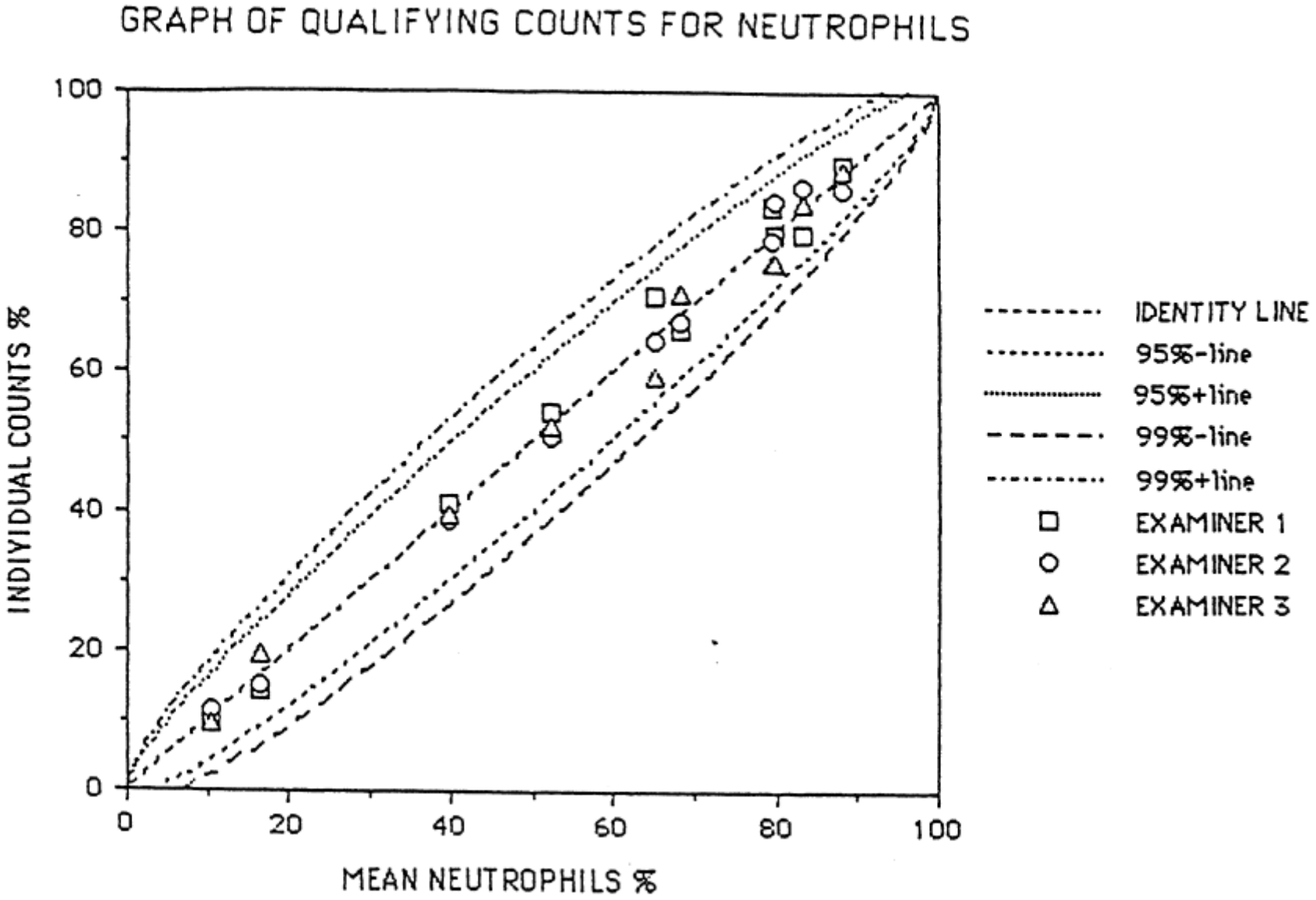
Basophils

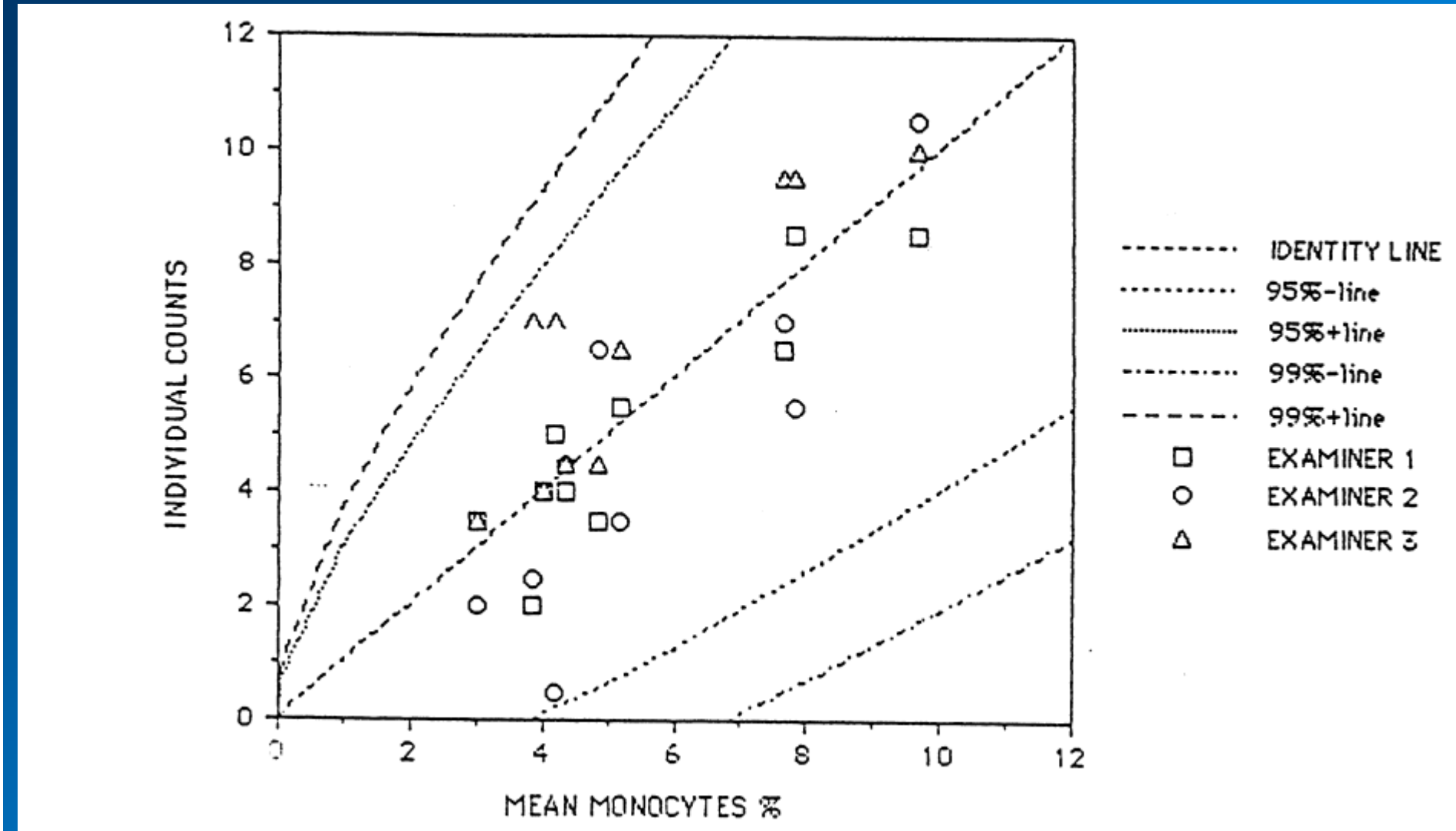
- (1) Basophilic granulocytes are smaller than eosinophils or neutrophils, usually 10 to 14 Fm in diameter.

- (2) They are characterized by densely stained, dark violet to purplish black cytoplasmic granules which are variably sized and unevenly distributed. Some granules usually overlay or even partially obscure the nucleus. The granules are water-soluble and therefore only vestiges of granules, sometimes apparently contained within small vacuoles, may be found.

- (3) The nuclei are deeply indented or segmented.

FIGURE 2


GRAPH OF QUALIFYING COUNTS FOR NEUTROPHILS
INDIVIDUAL COUNTS %
MEAN NEUTROPHILS %
0
20
40
60
80
100
IDENTITY LINE
95%-line
95%+line
99%-line
99%+line
EXAMINER 1
EXAMINER 2
EXAMINER 3


INDIVIDUAL COUNTS
MEAN MONOCYTES %
0
2
4
6
8
10
12
IDENTITY LINE
95%-line
95%+line
99%-line
99%+line
EXAMINER 1
EXAMINER 2
EXAMINER 3

Standard error of Proportion:

$$SE_p = \left[\frac{p \times q}{n}\right]^{\frac{1}{2}}$$

95% Confidence Interval for a single proportion:

$$p \pm 1.96\left[\frac{p \times q}{n}\right]^{\frac{1}{2}}$$

99% Confidence Interval for a single proportion:

$$p \pm 2.57\left[\frac{p \times q}{n}\right]^{\frac{1}{2}}$$

where:

n = 200 (cells observed per examiner)

p = mean value (of two or more examiners)

q = 100 - p

Let Student factor (S_f) be the 95th (or 99th) percentile of the t distribution with 199 degrees of freedom.

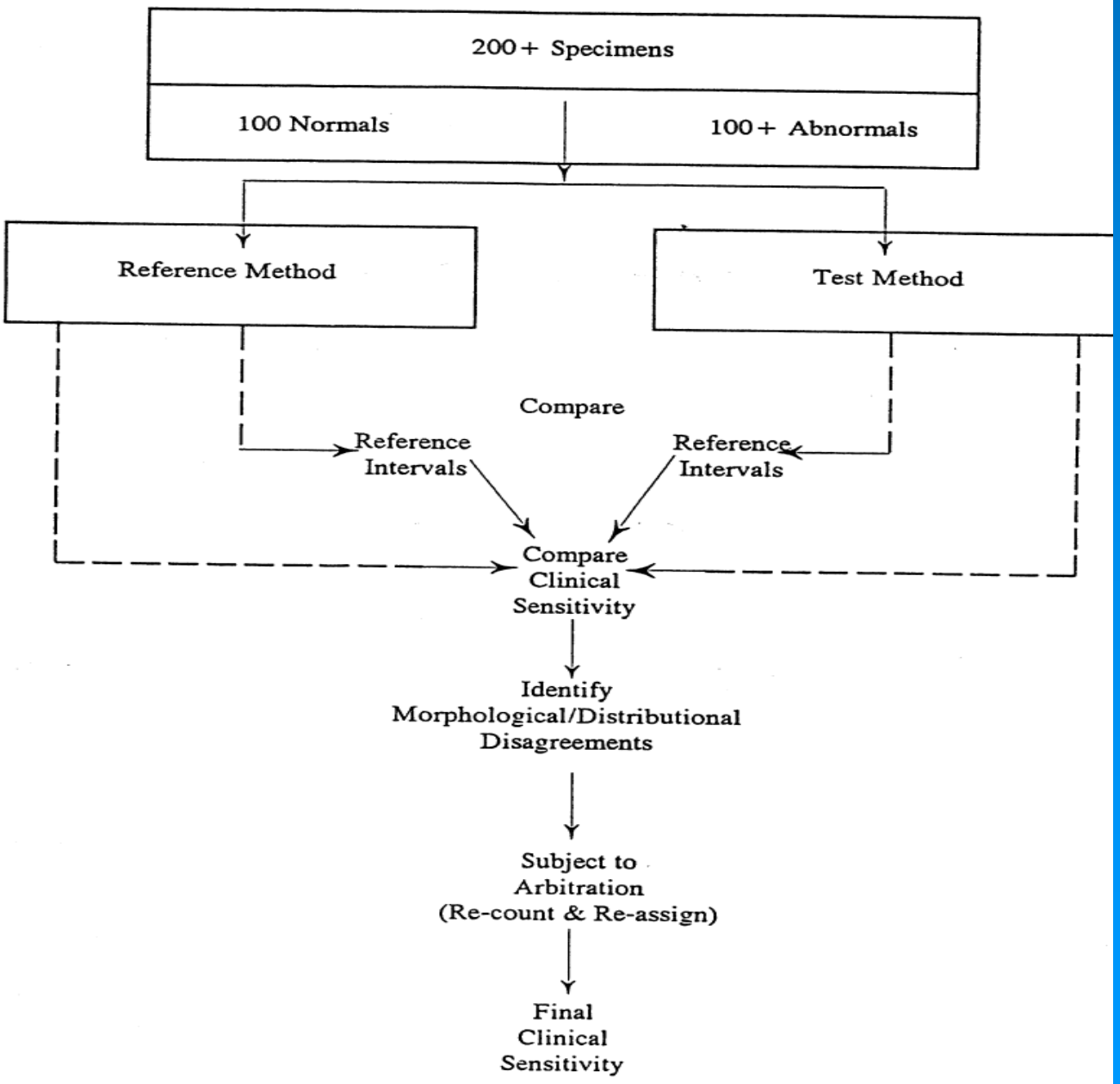
For a 95% confidence limit use $S_f = 1.96$
For a 99% confidence limit use $S_f = 2.57$.

An alternate method based upon the statistical studies of Rümke can be used to delineate the appropriate confidence bands.

95% AND 99% CONFIDENCE BANDS DERIVED FROM SEp

% CELLS	p	q	SEp	95% SEp	99% SEp	LOW 95%	HIGH 95%	LOW 99%	HIGH 99%
0	0	0	0	0	0	0	0	0	0
1	1	99	1	1	2	0	2	0	3
2	2	98	1	2	3	0	4	0	5
4	4	96	1	3	4	1	7	0	8
6	6	94	2	3	5	3	9	1	11
8	8	92	2	4	5	4	12	3	13
10	10	90	2	4	6	6	14	4	16
12	12	88	2	5	6	7	17	6	18
14	14	86	2	5	7	9	19	7	21
16	16	84	3	5	7	11	21	9	23
18	18	82	3	5	7	13	23	11	25
20	20	80	3	6	8	14	26	12	28
25	25	75	3	6	8	19	31	17	33
30	30	70	3	6	9	24	36	21	39
35	35	65	3	7	9	28	42	26	44
40	40	60	3	7	9	33	47	31	49
45	45	55	4	7	9	38	52	36	54
50	50	50	4	7	10	43	57	40	60
55	55	45	4	7	9	48	62	46	64
60	60	40	3	7	9	53	67	51	69
65	65	35	3	7	9	58	72	56	74
70	70	30	3	6	9	64	76	61	79
75	75	25	3	6	8	69	81	67	83
80	80	20	3	6	8	74	86	72	88
85	85	15	3	5	7	80	90	78	92
90	90	10	2	4	6	86	94	84	96
95	95	5	2	3	4	92	98	91	99
100	100	0	0	0	0	100	100	100	100

Outline of Experimental Protocol for Clinical Sensitivity



Specimen Types for Clinical Sensitivity Study

Clinical Condition	Characteristic Leukocyte Differential Count Finding	Absolute Cell Count	Proportional Cell Count*
Acute inflammation Bacterial infection	Granulocytosis and/or Left shift** (band-forms)	≥ 9.0 x 10^9/L ≥ 0.9 x 10^9/L	> 80% > 6%
Chronic inflammation	Monocytosis	≥ 0.8 x 10^9/L	> 10%
Parasitic infection Allergic reaction	Eosinophilia	≥ 0.5 x 10^9/L	> 7%
Viral infections infectious mononucleosis cytomegalovirus infection infectious hepatitis	Lymphocytosis and/or Lymphocytes, variant forms**	≥ 3.5 x 10^9/L ≥ 0.7 x 10^9/L	> 50%
Aplastic anemia, chemotherapy	Granulocytopenia	≤ 1.5 x 10^9/L	< 10%
HIV infection	Lymphopenia	≤ 1.0 x 10^9/L	< 7%
Acute leukemia	Immature cells, including blasts**	≥ 0.1 x 10^9/L	> 2%
Severe anemia myelophthisic anemia	Nucleated red blood cells**	≥ 0.02 x 10^9/L	> 2%

* In addition to noted absolute counts, the specimens should also have these proportional counts.

** Findings for morphological classification; other findings are considered to be distributional changes. Aim to include at least five cases of each condition in the clinical sensitivity study.


so thanks