Manual Methods

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Today's Discussion

- Criteria for Manual Methods
- Manual Cell Counts
  - WBC
  - Platelet
- Measuring Hemoglobin
- Microhematocrit
- Rule of Three
- Manual Reticulocyte Count
- Case Studies

Manual Method

Criteria for Manual Method

- May be necessary when counts exceed the linearity of an instrument
- Instrument is nonfunctional and there is no backup
- Remote laboratories in Third World countries
- Disaster situation when testing is done in the field

Manual Counts

Why perform the manual count?

- Ensure the accuracy of the automated counts
- If many giant platelets are present on a smear
- Both WBC and PLT estimate appear higher or lower than the instrument results

Performed using a hemocytometer or counting chamber

Manual dilutions maybe performed using calibrated, automated pipettes and diluents

Cell count is essentially the same for WBCs, RBCs, and platelets

- Dilution, diluting fluid, and area counted may vary

Hemocytometer

- Two raised surfaces, each with a 3mm x 3mm square counting area or grind (total area 9mm^2)
- Separated by an H-shaped moat
- Grid made up of nine 1mm X 1mm squares
- Each of the 4 corner (WBC) squares subdivided further into 16 squares
- Center square is subdivided into 25 smaller squares
  - Each square is 0.2 mmx 0.2mm
    - 1/25 of the center square or 0.04 mm^2
- Coverslip is placed on top of the counting surface
  - Distance between counting surface and coverslip is 0.1 mm
- Total volume of one entire grid or counting area on 1 side of the hemocytometer is 0.9 mm^3

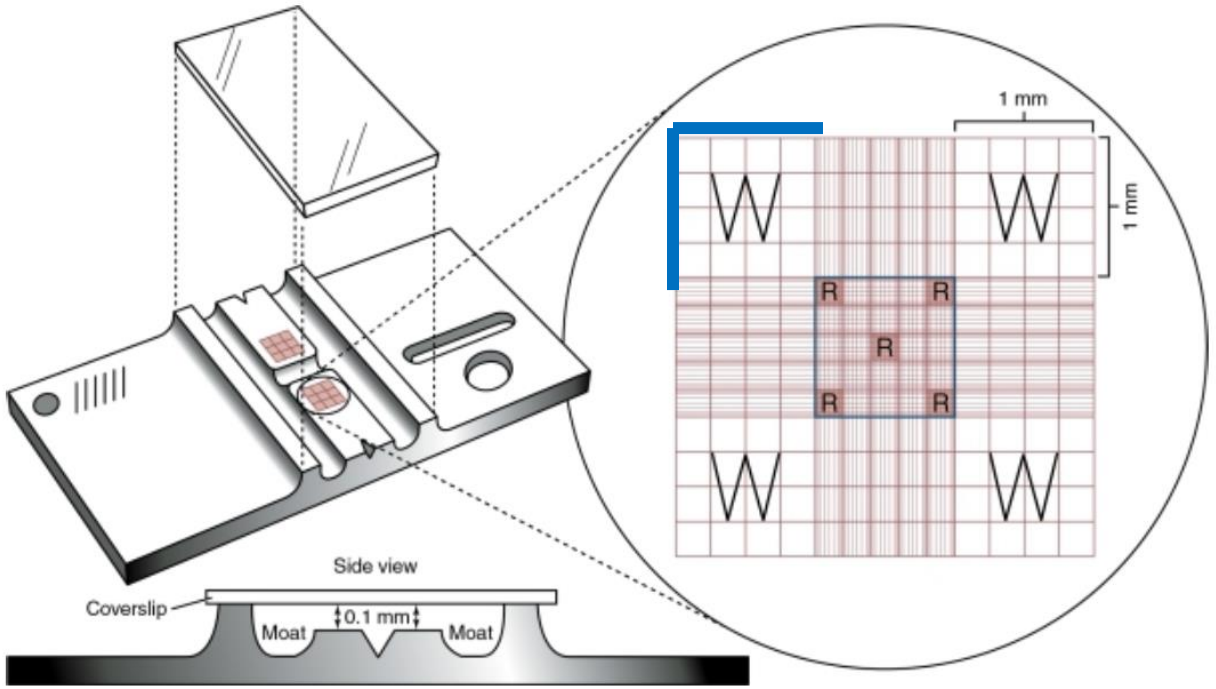


Figure 11.1**Hemacytometer and Close-up View of the Counting Areas as Seen Under the Microscope.** The areas for the standard white blood cell count are labeled W, and the areas for the standard red blood cell count are labeled R. The entire center square, outlined in blue, is used for counting platelets. The side view of the hemacytometer shows a depth of 0.1 mm from the surface of the counting grid to the coverslip.

Rodak's Hematology 6th edition

Hemocytometer Calculation

- General Formula:

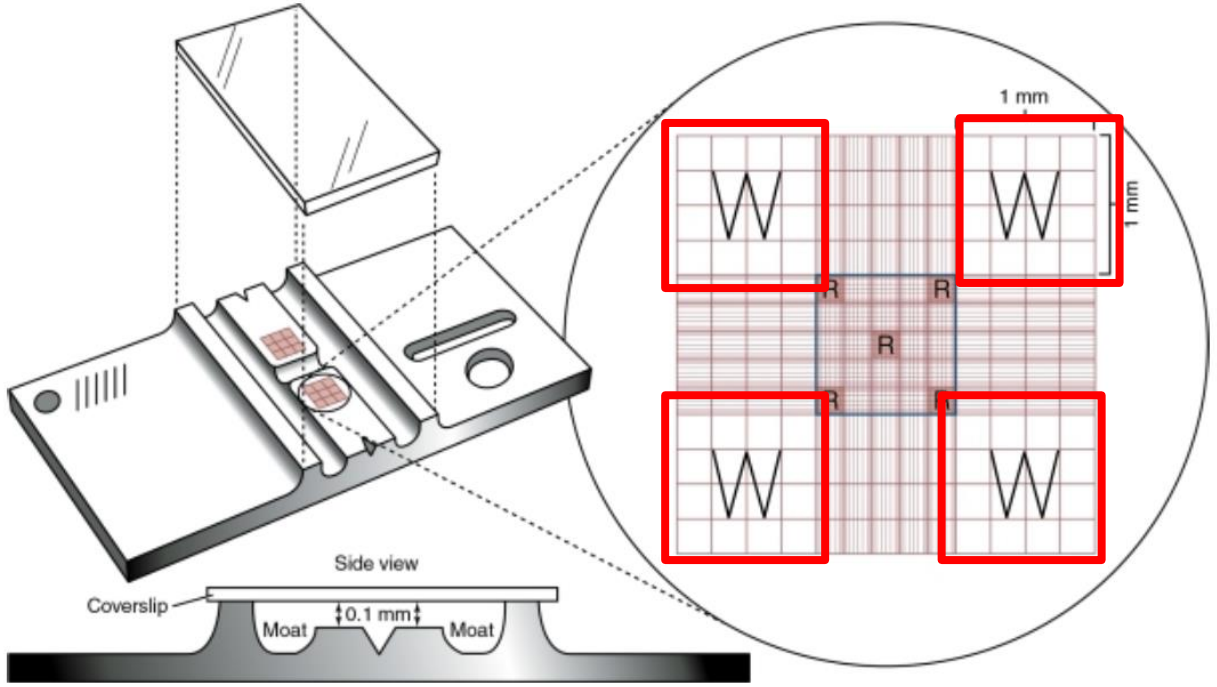
$$\text{Total Count} = \frac{Cells\ Counted(Average)\ x\ Dilution\ Factor}{Area}$$

- Yields the number of cells per mm^3

Hemocytometer

WBCs Count

- Dilution:
  - Whole blood from an EDTA tube is diluted with 1% buffered ammonium oxalate or a Weak acid solution (3% acetic acid or 1% hydrochloric acid)
  - The dilution will lyse the nonnucleated RBCs in the specimen (preserves WBCs and PLTs)
  - Typical dilution- 1:20 or 1:100
- Count Area:
  - 4 large corner squares
    - 4 mm^2
  - Entire counting area (all 9 squares)
    - 9 mm^2



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WBC Hemocytometer Calculation

Example: Side 1: 92 and Side 2: 100

- Difference between the side is <10%
- Average= 96
- Dilution: 1:20
- Area counted: 4 large squares (4 $mm^{2)}$

$$\text{WBC Count} = \frac{cells\ counted\ (average) x\ dilution\ factor}{area}$$

$$\text{WBC Count} = \frac{96\ x20}{4\ x\ 0.1}$$

WBC Count= $4800/mm^3$ or $4.8 \times 10^3/\mu L$

WBC Manual Count Sources of Error

- Hemocytometer and coverslip should be cleaned properly before they are used
  - Dust and fingerprints may cause difficulty in distinguishing the cells
- Diluting fluid should be free of contaminants
- If the count is low, a greater area may be counted (e.g. 9 mm^2) to improve accuracy
- Chamber must be charged properly, uneven flow of the diluted blood can result in an irregular distribution of cells

WBC Manual Count Sources of Error

- Any nucleated RBCs (NRBCs) present in a specimen cannot be lysed by the diluting fluid and are indistinguishable from WBCs on a hemocytometer
  - If 10 or more NRBCs per 100 WBCs are observed on the differential count on a stained PB smear, the WBC count must be corrected for these results
  - Accomplished through the following formula

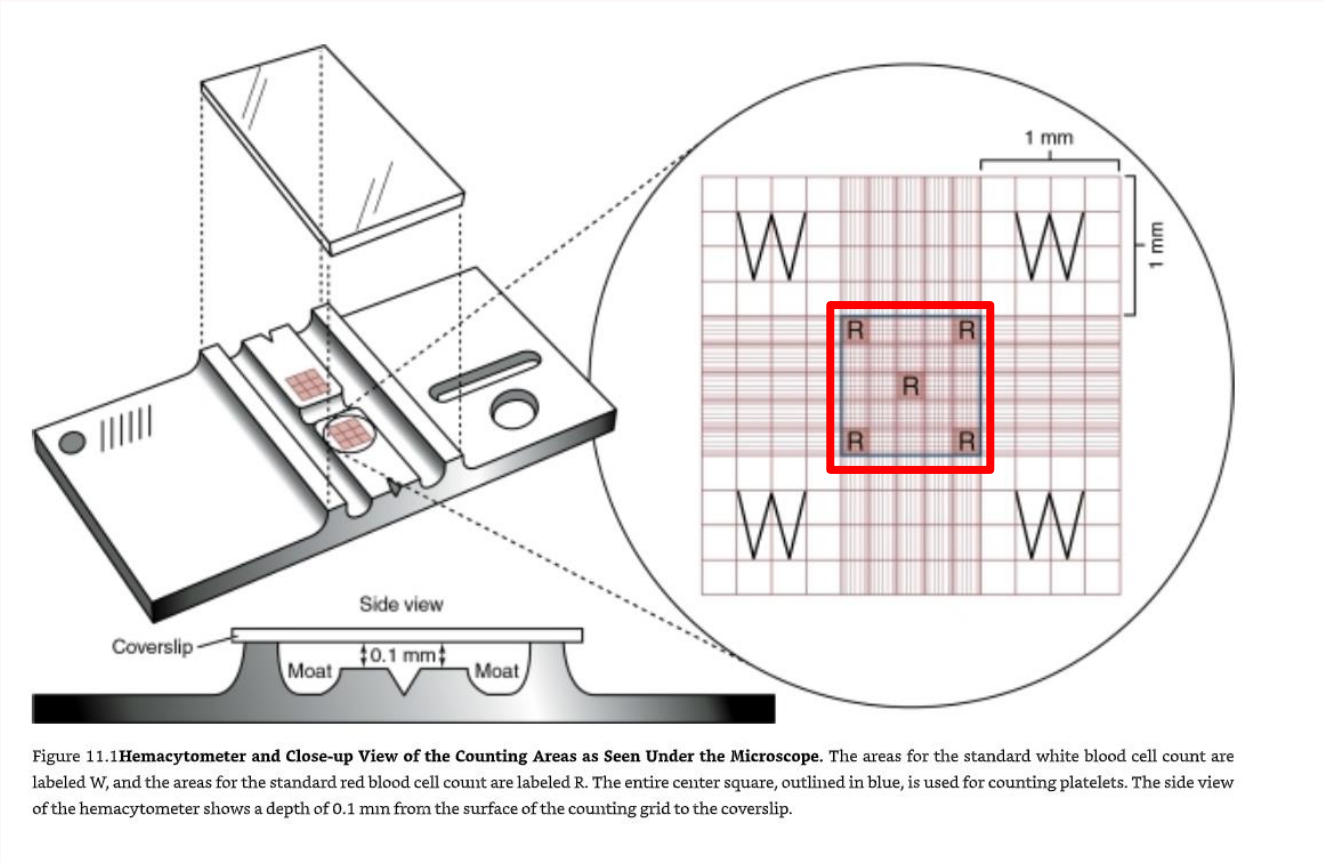
$$\frac{Uncorrected\ WBC\ count\ x\ 100}{Number\ of\ NRBCs\ per\ 100\ WBCs + 100}$$

  - Report the result as the "corrected" WBC count
  - Accuracy of the manual WBC count can be assessed by performing a WBC estimate on a wright-stained peripheral blood smear

Hemocytometer

Platelet Count

- Dilution:
  - 1:100 with 1% ammonium oxalate
    - Lyse the nonnucleated RBCs
- Counted in the 25 small squares in the larger center square (1 mm^2) of the hemocytometer
- Phase-contrast microscope is the reference method
- Light microscope can be used but visualizing the platelets might be difficult
- Round or oval with diameter of 2-4 µm



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PLT Hemocytometer Calculation

Example: Side 1: 198 and Side 2: 202

- Difference between sides is <10%
- Average: 200
- Dilution: 1:100
- Area Counted: 1 square counted (1 mm^2)

$$\text{PLT Count} = \frac{Cells\ Counted\ (Average) x\ Dilution}{Area}$$

$$\text{PLT Count} = \frac{200\ x\ 100}{1\ x\ 0.1}$$

PLT Count= 200,000/mm^3 or 200 x $10^3/\mu L$

PLT Manual Count Sources of Error

Improper collection can cause the platelets to clump on the hemocytometer
- try to resolve the issue with a new dilution or specimen recollection

Dirt in the pipette, hemocytometer, or diluting fluid may cause the count to be inaccurate

Adjustment to dilutions used:
- <50 platelets are counted on each side
  - Repeat the count using a lower dilution (1:20)
- >500 platelets are counted on each side
  - Repeat the count using a greater dilution (1:200)

"Platelet satellitosis"
- Refers to the adherence of platelets around neutrophils
- Can occur if EDTA anticoagulant is used
- Can be corrected through the use of sodium citrate as the anticoagulant
  - Necessary to multiple the platelet count by 1.1 to correct for the dilution in the citrate evacuated tubes

Measuring Hemoglobin

Measuring Hemoglobin

- HGB concentration can be determined by measuring its color, by its power of combining with oxygen or carbon monoxide, or by its iron content

- Cyanmethemoglobin
  - Main method

- Alternative methods:
  - Sodium lauryl sulfate
  - Methemoglobin

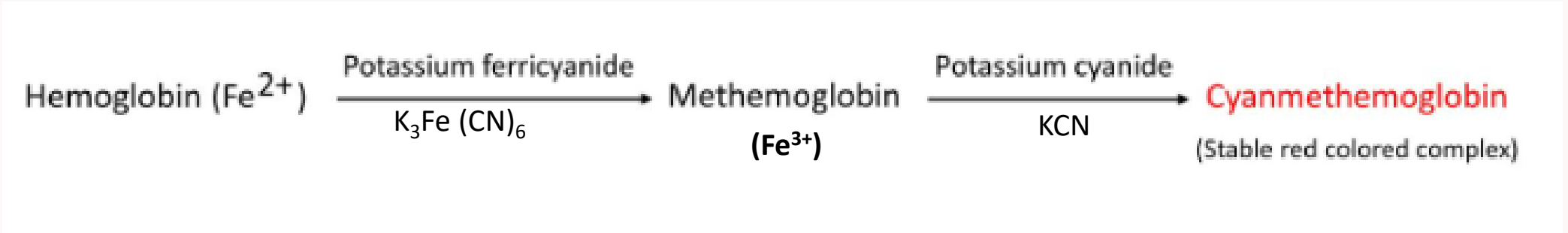
Cyanmethemoglobin Method

Principle:

- Measurement of hemoglobin by color
- Drabkin solution is mixed with whole blood which converts hemoglobin → stable cyanmethemoglobin
- Color intensity of cyanmethemoglobin is measured at 540 nm and compared to a known standard
  - Directly proportional to the hemoglobin concentration

Drabkin Reagent

- Potassium ferricyanide, potassium cyanide, sodium bicarbonate, and a surfactant



https://laboratorytests.org/cyanmethemoglobin-method/

Cyanmethemoglobin Method

- Cyanmethemoglobin reagent is sensitive to light, should be stored in a brown bottle in a dark place
- Cyanmethemoglobin reagent contains cyanide- highly toxic
- Carboxyhemoglobin takes 1 hour to convert to cyanmethemoglobin
  - Theoretically can cause erroneous results in specimens from heavy smokers
- Sulfhemoglobin cannot be converted to cyanmethemoglobin
  - Cannot be measured by this method
  - Seldom encountered in clinical practice

Sources of Error

- High WBC count or high platelet count
  - Causes turbidity and a falsely high result
  - Reagent-specimen solution can be centrifuged and supernatant measured
- Lipemia
  - Cause turbidity and a falsely high result
  - Corrected by adding 0.01 mL of patient's plasma to 5 mL of cyanmethemoglobin reagent and using this solution as a blank
- Presence of Hb C and Hb S
  - Resistant to hemolysis and cause turbidity
  - Corrected by making a 1:2 dilution with distilled water and multiplying the standard curve by 2
- Abnormal globulins which precipitate
  - May precipitate in the reagent
  - Corrected by adding 0.1 g of potassium carbonate to the cyanmethemoglobin reagent

Alternative Methods to Measure Hb

- Sodium lauryl sulfate (SLS)
  - Used in many automated analyzers
  - Convert hemoglobin to SLS-methemoglobin
  - Does not generate toxic waste
- Methemoglobin
  - Measured by spectral absorption analysis instrument such as the CO-oximeter
    - Methemoglobin shows an absorption peak at 630 nm

Microhematocrit

Microhematocrit

- Hematocrit- volume of packed RBCs that occupy a given volume of blood
- EDTA or heparin anticoagulant
- Steps:
  - Fill 2 plain capillary tubes ¾ full of blood
  - Seal the end of the tube with the colored ring using nonabsorbent clay, approx. 4 mm long
  - Balance the tubes in a microhematocrit centrifuge with clay ends sticking out
  - Centrifuged at 12500 RCF for 8 minutes to obtain maximum packing of RBCs
  - Determine the hematocrit using the microhematocrit reading device
  - Duplicate values should match within 1% (0.01 L/L)

Figure 11.7 **Microhematocrit Reader.**

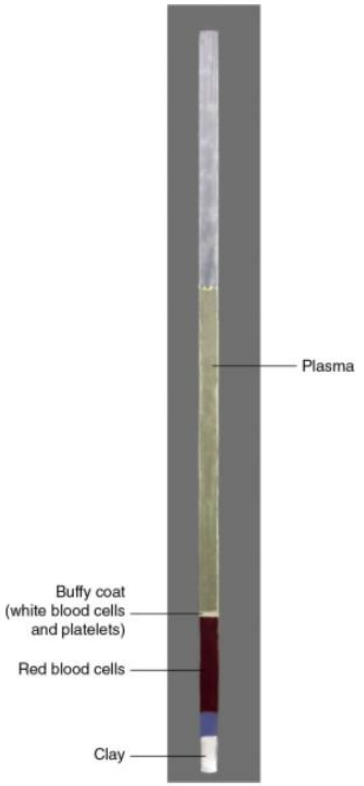


Figure 11.8 **Capillary Tube with Anticoagulated Whole Blood after Centrifugation.** Notice the layers containing

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Sources of Error

- Improper sealing of the tubes can cause a decreased hematocrit
- Increased concentration of anticoagulant decreases the hematocrit reading because of RBC shrinkage
  - Short draw in an evacuated tube
- If the buffy coat is included in the reading, this will cause a falsely elevated result
- Oddly shaped RBCs will cause plasma to be trapped in the RBC layer and cause the microhematocrit to be 1-3% higher than the value obtained using a automated instrument
  - Sickle cell anemia, macrocytic anemias, hypochromic anemias, spherocytosis, and thalassemia
- Decreased hematocrit seen after blood loss since plasma is replaced faster than RBCs
- Dehydration causes a increased hematocrit (less plasma)
- Interstitial fluid from a skin puncture causes decreased hematocrit readings

Rule of Three

Rule of Three

Relationship between the RBC count, hemoglobin, and hematocrit

Used to perform a quick visual check of the results of the hemoglobin and hematocrit

Applies to normocytic, normochromic RBCs

Normally these 3 values follow the Rule of Three

- Hemoglobin should be 3x the RBC count
- Hematocrit should be 3x the hemoglobin (+/-3)

If the values do not agree, the blood film should be examined for abnormal RBCs and false increases/decreases in the hemoglobin or hematocrit should be investigated

Example: RBC= 4.50, HGB= 13.5, and HCT= 40.5

Manual Reticulocyte Count

Reticulocyte Count Principle

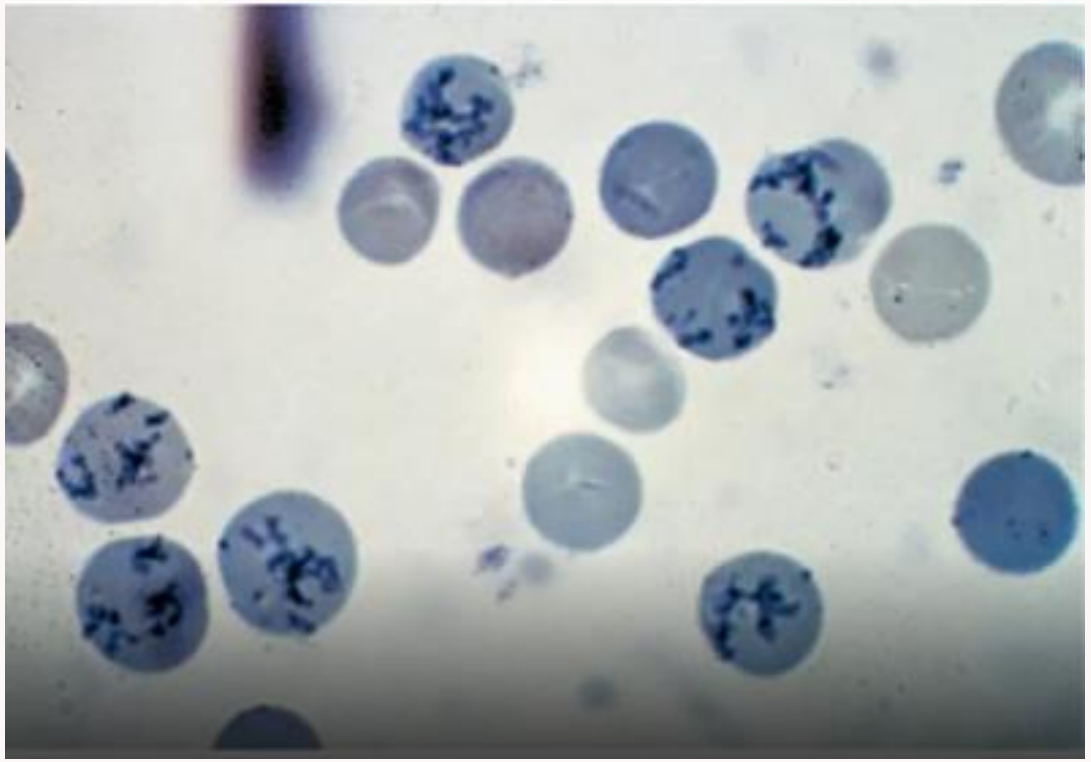
Reticulocyte

- Last immature RBC stage
- Contains remnant cytoplasmic ribonucleic acid (RNA) and organelles such as the mitochondria and ribosomes

Performing a reticulocyte count

- Whole blood, anticoagulated with EDTA, is stained with a supravital stain
  - Nucleic acid stains or vital stains
  - New methylene blue dyes

Any nonnucleated RBC that contains two or more particles of blue-stained granulofilamentous material after the staining is defined as a reticulocyte



https://www.bioscience.com.pk/topics/cell-biology/item/145-reticulocyte

Manual Retic Count

- Two wedge films are prepared from a blood-stain mixture
- Under oil immersion and an area where the RBCs are close but not touching 1000 RBCs are counted (500 from each of the slides)
  - The retic and total RBCs (retics and RBCs) are used in the following equation:

- Calculation:

$$\frac{\#\ of\ Reticulocytes\ x\ 100}{1000\ RBCs\ counted} = Reticulocytes\ (\%)$$

Manual Retic Count

Miller disc

- Used to reduce the labor-intensive process
- Disc composed of 2 squares
  - Area of the smaller square measuring 1/9 the area of the larger square
- Inserted into the eye piece of the microscope
- Count:
  - RBCs are counted in the smaller square
  - Retics are counted in the larger square
- Minimum of 112 RBCs should be counted in the small square (equivalent to 1008 RBCs)

$$\frac{\#\ of\ Retics\ in\ square\ A\ (large\ square) x 100}{\#\ RBCs\ in\ square\ B\ (small\ square) x\ 9} = \text{Retic \%}$$

Ex. 15 retics in the large square and 112 RBCs in the small square

$$\frac{15\ x\ 100}{112\ x\ 9} = 1.5\%$$

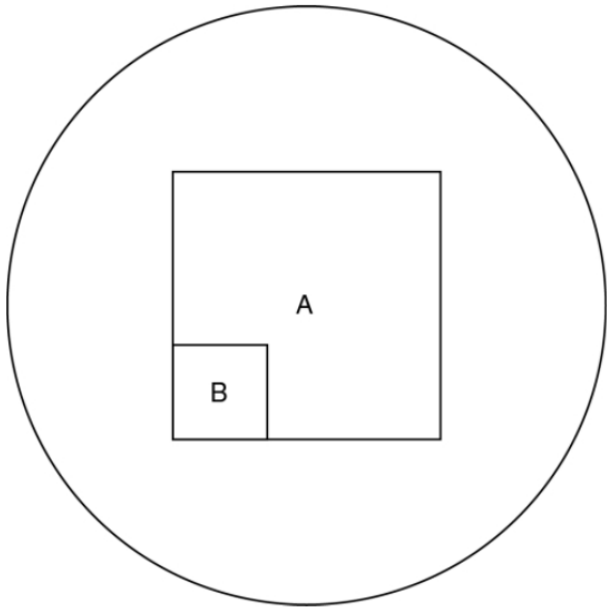


Figure 11.11 **Miller Ocular Disc Counting Grid as Viewed through a Microscope.** The area of square B is 1/9 the area of square A. Alternatively, square B may be in the center of square A.

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Sources of Error

- More or less blood to be added to stain for anemia or polycythemic patients
- Error may occur if the blood and stain are not mixed before the films are made
- Moisture in the air, poor drying of the slide, or both may cause areas of the slide to appear retractile and can be confused for retics
- Other RBC inclusions can be stained supravitally: Heinz Bodies, Howell-Jolly bodies, and Pappenheimer bodies
  - Heinz bodies- Precipitated hemoglobin, usually round/oval on the cell membrane
  - Howell-Jolly bodies- Round nuclear fragments, usually singular
  - Pappenheimer bodies

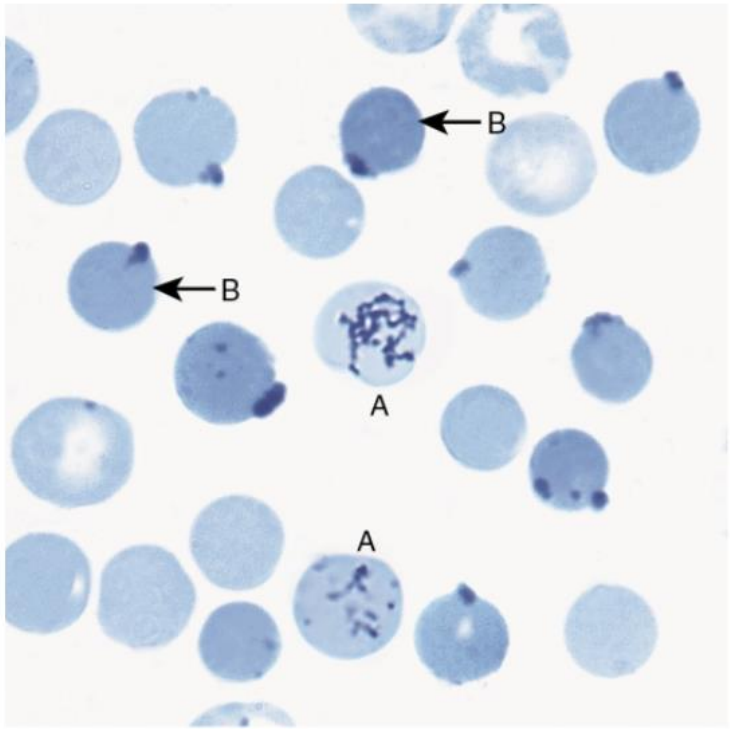

Figure 11.12**Reticulocytes and Heinz Bodies. (A),** Reticulocytes. **(B),** Heinz bodies. (Peripheral blood, New methylene blue vital stain, ×1000.)

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Absolute Reticulocyte Count (ARC)

- Actual number of reticulocytes in 1 L or µL of blood
- Calculation:

$$\text{ARC} = \frac{Retics\ (\%) x\ RBC\ count}{100}$$

- Example: patient's reticulocyte count is 2% and the RBC count is 2.20 x10^{12} /L

$$\frac{2\ x\ 2.20}{100} = 44 \times 10^{9}/L$$

Corrected Reticulocyte Count

- Needed for Specimens with low hematocrit/anemia patients
  - The % of retics may be falsely elevated
    - The whole blood contains fewer RBCs
- Correction factor must be used with the average normal hematocrit considered to be 45%
- Calculation:

$$\textbf{Corrected retic count (\%)} = \frac{\textit{reticulocycte}\ (\%) x\ \textit{patient hematocrit}\ (\%)}{45\ (\textit{average normal hematocrit})}$$

- Corrected Reticulocyte count depends on the degree of anemia

Reticulocyte Production Index (RPI)

- Reticulocytes that are released from the marrow prematurely are called **shift reticulocytes**
  - Shifted from the bone marrow to the PB earlier than usual to compensate for anemia
  - Require more than 1 day to mature
    - Take 2 to 3 days to lose their reticula
    - Will contribute to the reticulocyte count for more than 1 day

Shift reticulocytes result in a falsely increased reticulocyte count

- As the severity of anemia increases, the number of days maturing the blood increases

Reticulocyte Production Index (RPI)

Patient's Hematocrit Value (%)	Correction Factor (Maturation Time, Days)
40–45	1
35–39	1.5
25–34	2
15–24	2.5
< 15	3

$$\text{RPI} = \frac{Corrected\ Reticulocyte\ Count}{Maturation\ Time}$$

Example: Retic count= 7.8%, Hct= 30%, polychromasia noted

-Table shows correction factor of 2

$$\text{RPI} = \frac{7.8x\ (^{30}/_{45})}{2}$$

RPI= 2.6

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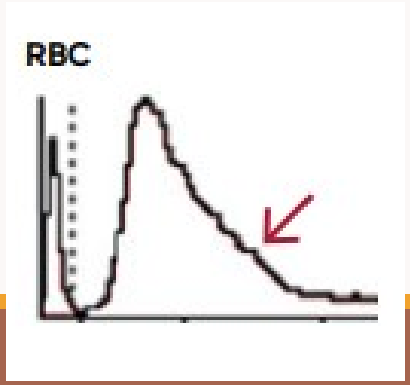
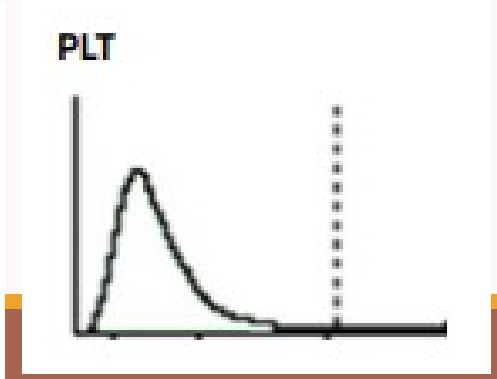
Case Studies

Case Study #1

Parameter		Ranges
WBC	5.82	3.6-10.6 x10^3/ µL
RBC	0.82 ↓	4.0-6.0 x 10^6/ µL
HCT	9.4 ↓	35-50%
MCV	114.6 ↑	76-100 fL
MCH	104.9 ↑	26-34 pg
MCHC	91.5 ↑	32-36 g/dL
PLT	180	150-450

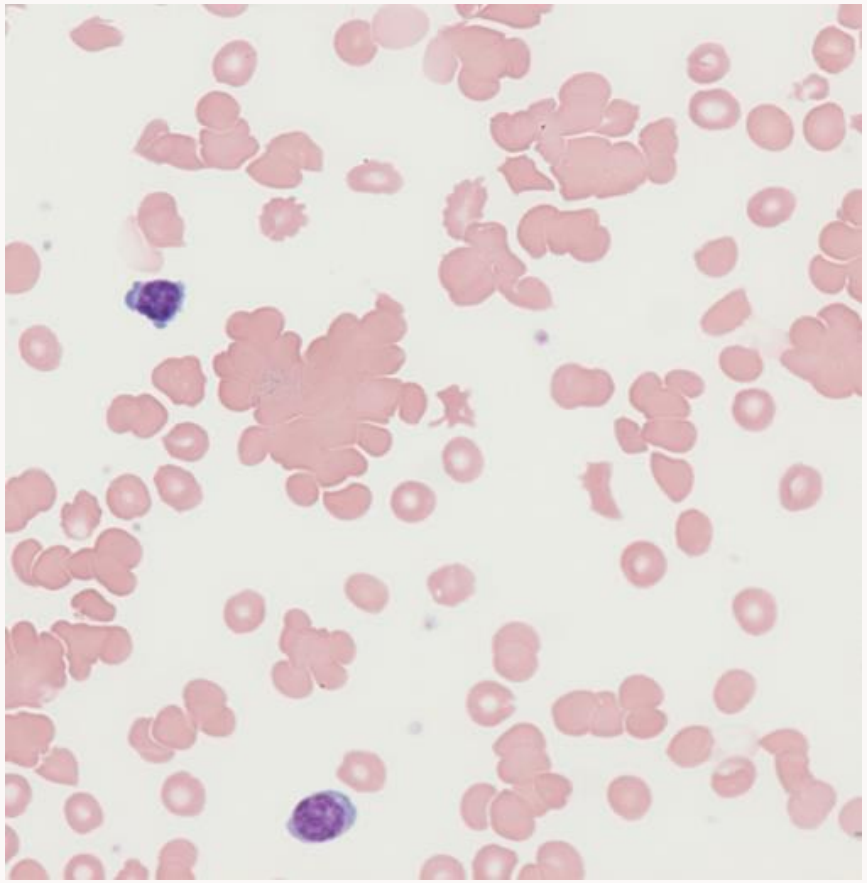




- Results
  - Increased MCV, MCH, MCHC
  - Decreased RBC, HCT
  - Right shift on RBC histogram
- Potential Cause of results
- Okay to validate? Is corrective action needed?

https://www.thebloodproject.com/cold-agglutinin-disease-2/

Case Study #1



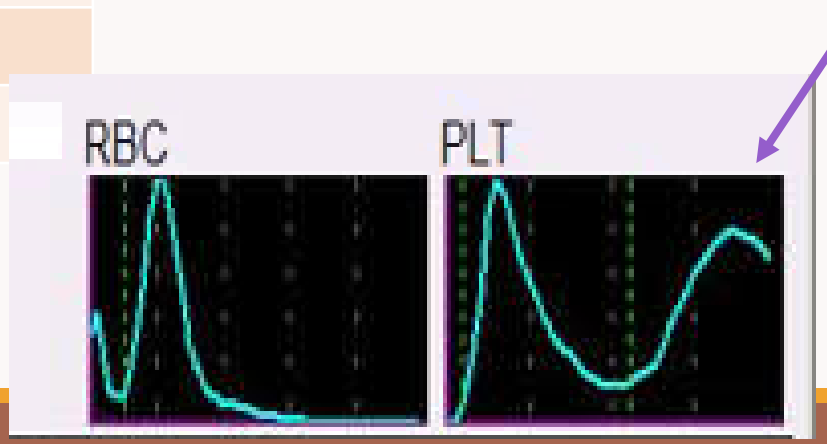
- Results
  - Increased MCV, MCH, MCHC
  - Decreased RBC, HCT
  - Right shift
- Potential Cause of results
  - Cold Agglutinin
- Corrective Action
  - Incubate specimen at 37 C for 15 minutes
  - Rerun specimen

https://www.thebloodproject.com/cold-agglutinin-disease-2/

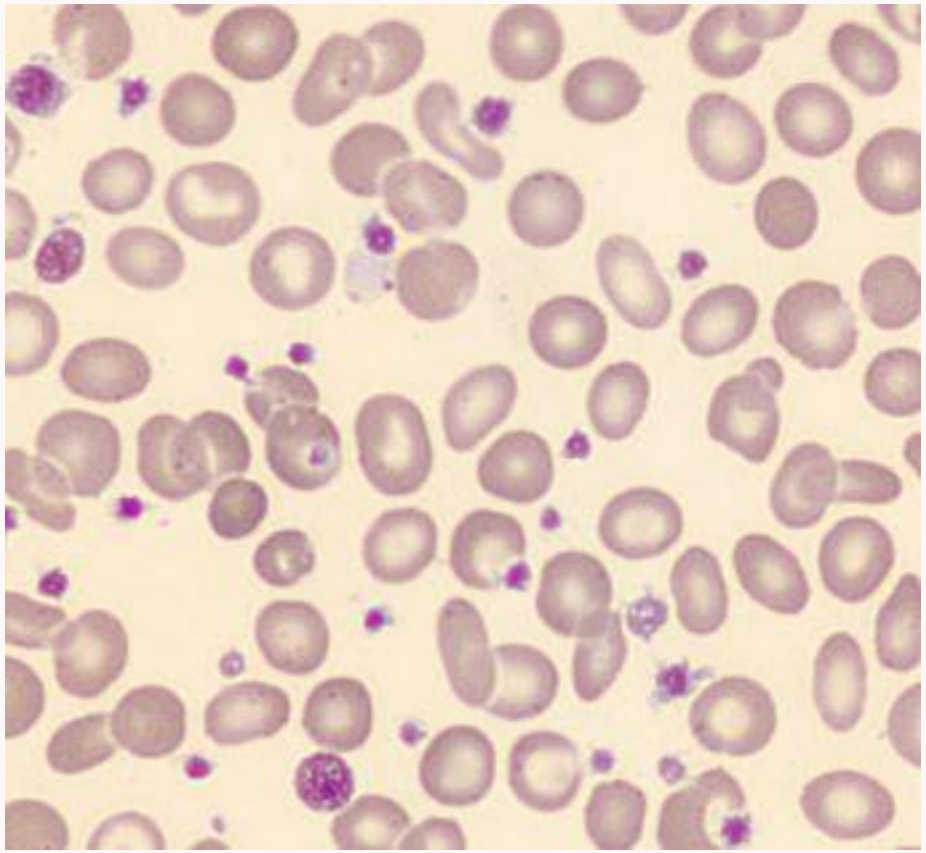
Case Study #2

Parameter		Ranges
WBC	18.42 ↑	3.6-10.6 x10^3/ µL
RBC	6.28 ↑	4.0-6.0 x 10^6/ µL
HCT	38.6	35-50%
MCV	61.5 ↓	76-100 fL
MCH	17.2 ↓	26-34 pg
MCHC	28.0 ↓	32-36 g/dL
PLT	134 ↓	150-450
RDW	24.2 ↑	11-15%

- Results:
  - Increased WBC, RBC, RDW
  - Decreased PLT, MCV, MCHC, MCH
  - Abnormal PLT histogram
- Peripheral Blood Smear Features
- Resolution



Case Study #2



- Results:
  - Increased WBC, RBC, RDW
  - Decreased PLT, MCV, MCHC, MCH
  - Abnormal PLT histogram
- Peripheral Blood Smear Features
  - Giant platelets
  - Red Blood Cells
    - Hypochromic
    - Large central pallor
    - Anisocytosis
    - RBC abnormalities
- Resolution
  - Platelet estimate from smear or manual platelet count

References

- Rodak's Hematology 6th Edition
- Pinal Patel Laboratory Education Specialist