CYTOCHEMICAL STAINING IN HAEMATOLOGY

MANSI DAMECHANI

HAEMATOLOGY LABORATORY

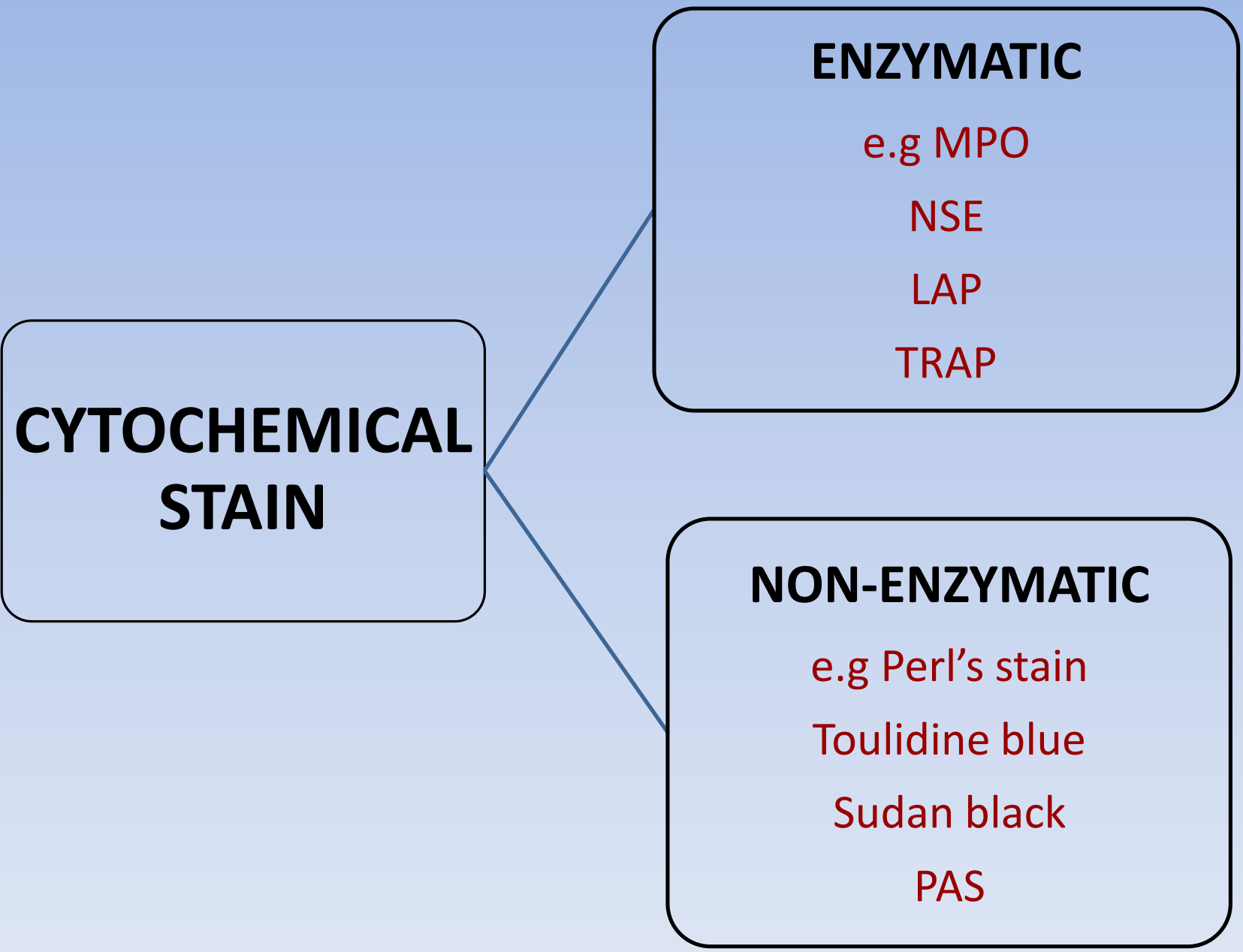
TATA MEMORIAL HOSPITAL

WHAT IS CYTOCHEMICAL STAIN

- CYTO MEANS CELL
- CHEMICAL MEANS BIOCHEMICAL REACTIONS WHICH OCCUR INSIDE THE CELL
- STAIN MEANS TO VISUALISE THESE REACTIONS WITH THE HELP OF COLOURING DYE

CYTOCHEMICAL STAIN

- Can be performed on peripheral blood smears and bone marrow smears.
- They play a very important role in diagnosis, differentiation and classification of Leukemia (FAB classification)
- Preliminary Screening Tool (In absence of Advanced Techniques)
- Detecting cytoplasmic abnormalities and enzyme deficiencies in myeloid disorder
- Cheap and cost effective, less time consuming


CYTOCHEMICAL STAIN
ENZYMATIC
e.g MPO
NSE
LAP
TRAP
NON-ENZYMATIC
e.g Perl's stain
Toulidine blue
Sudan black
PAS

Cytochemical stain

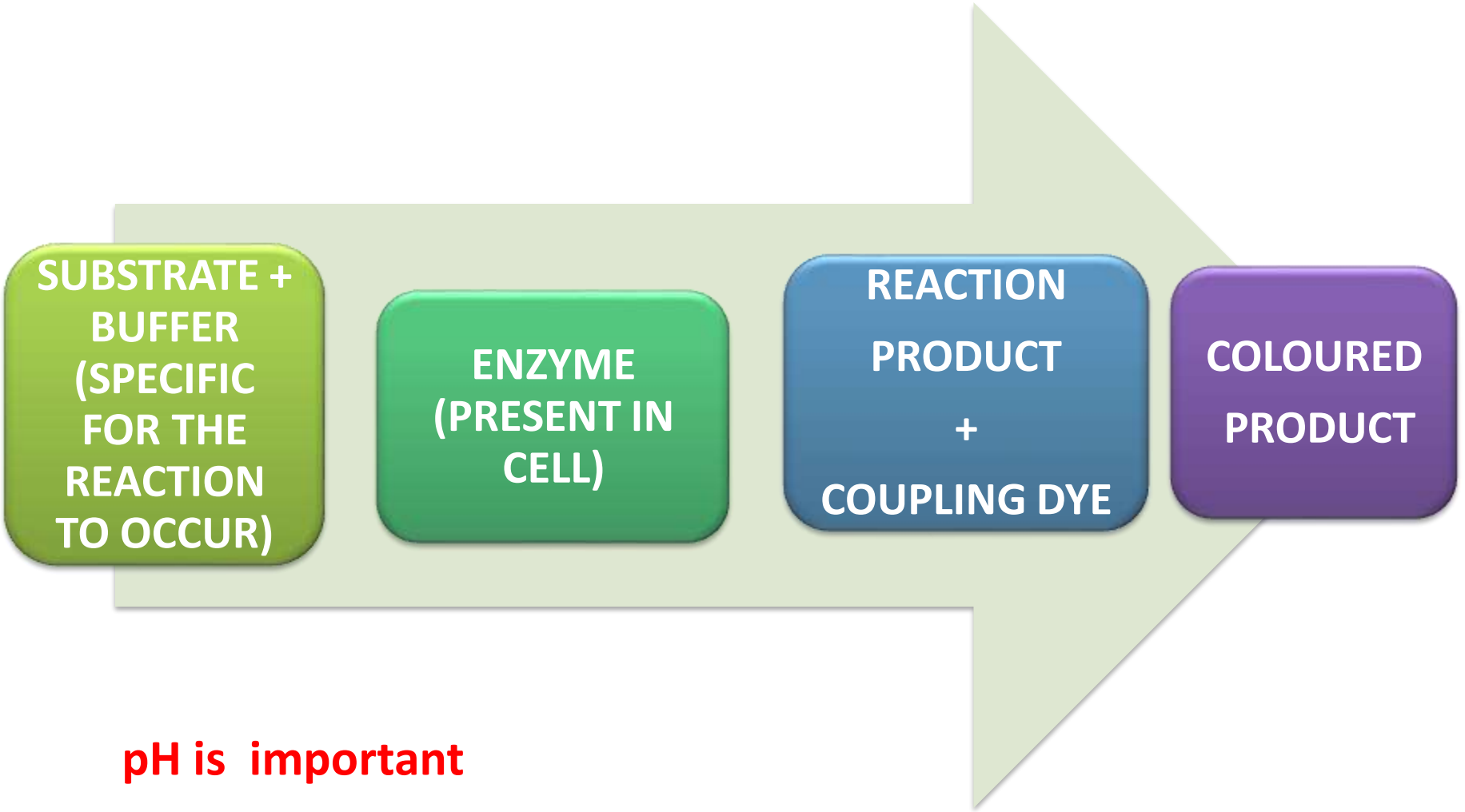
WBC

- MPO
- LAP
- Esterases
- TRAP
- Sudan Black B
- PAS
- Toluidine blue

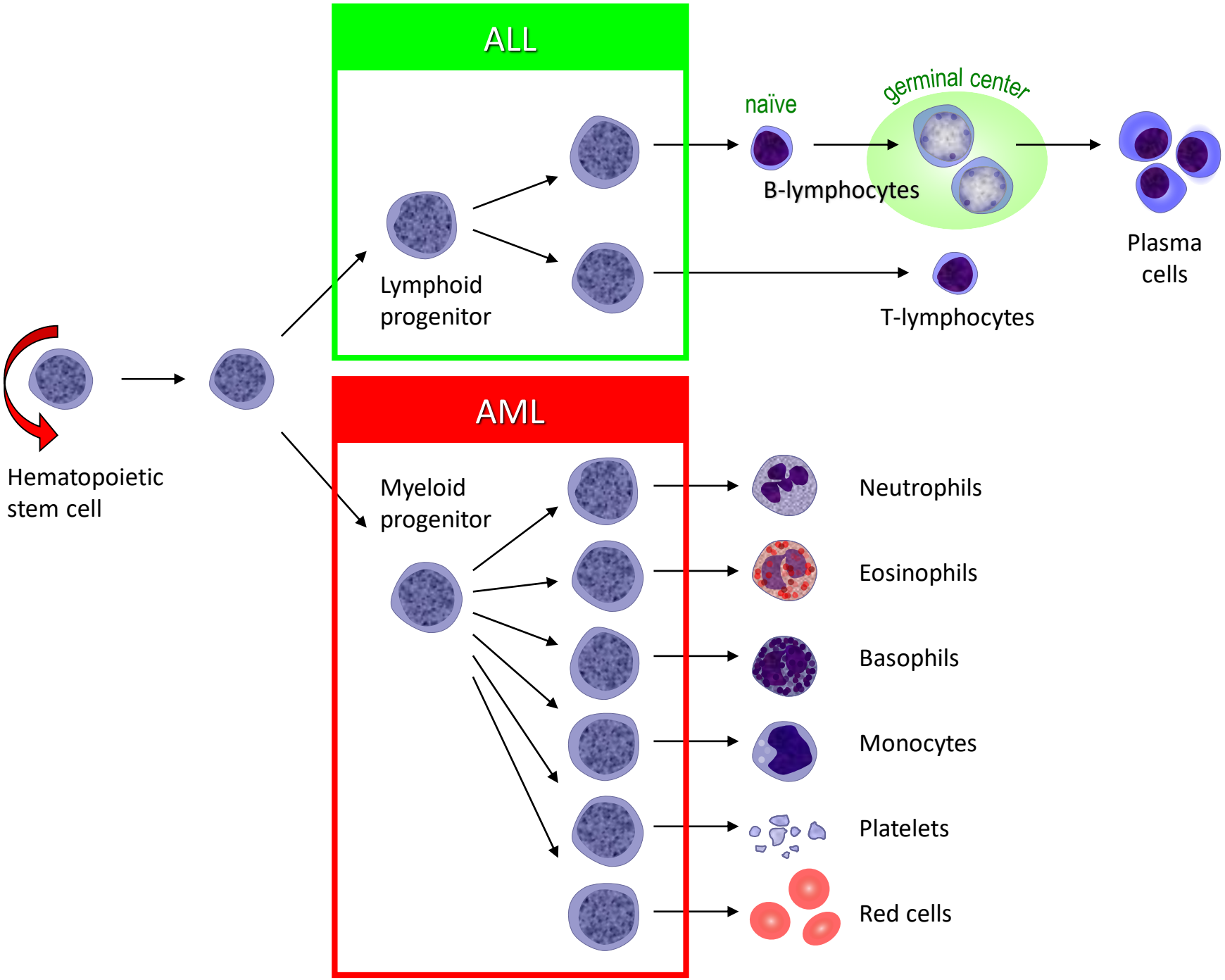
RBC

- Perl's stain or Iron stain

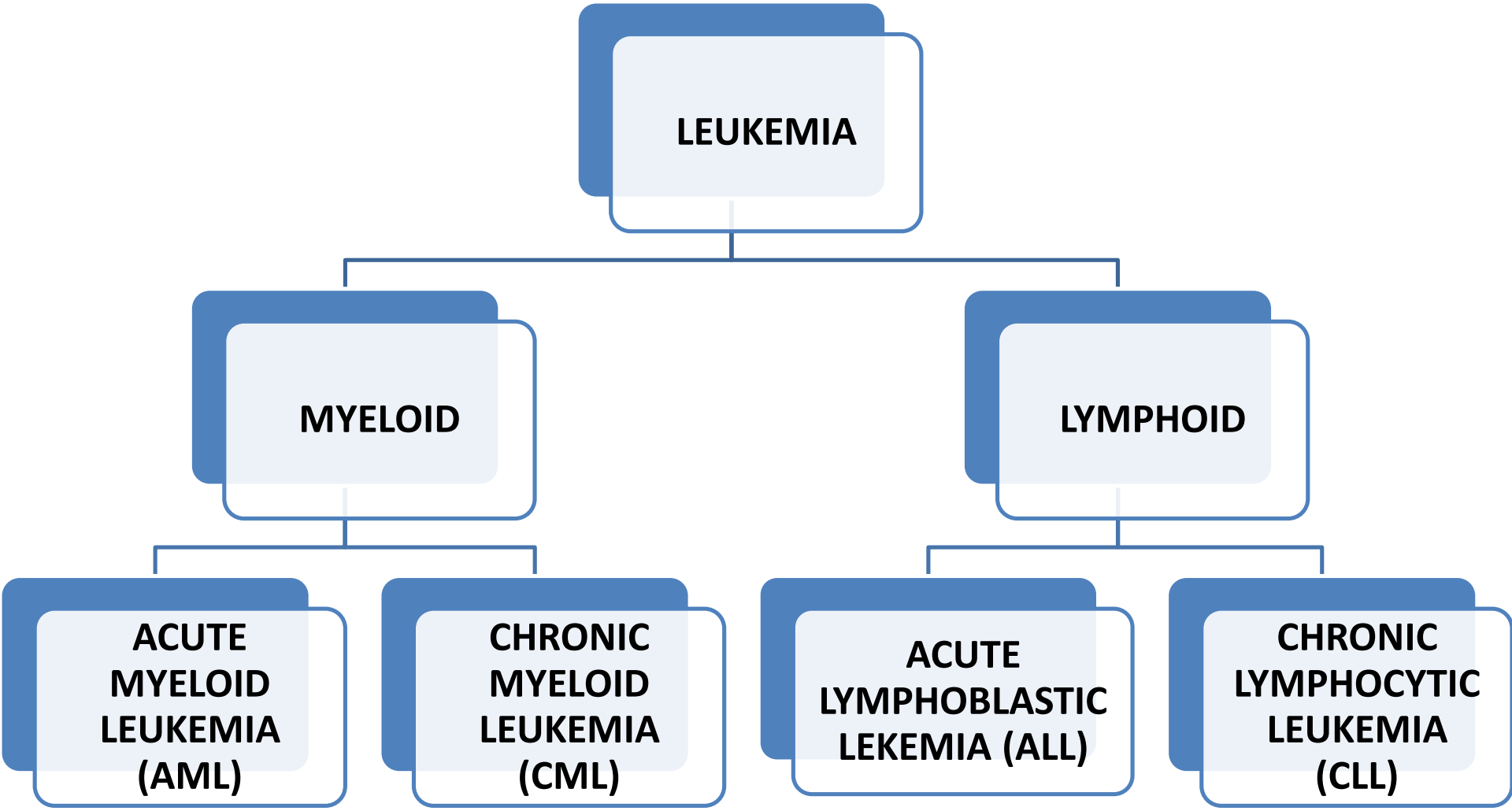
HOW REACTIONS OCCUR IN ENZYME BASED STAIN ???



pH is important


ALL
naïve
germinal center
B-lymphocytes
Plasma cells
Lymphoid progenitor
T-lymphocytes
Hematopoietic stem cell
AML
Myeloid progenitor
Neutrophils
Eosinophils
Basophils
Monocytes
Platelets
Red cells

CLASSIFICATION OF LEUKEMIA



Cytochemical stain

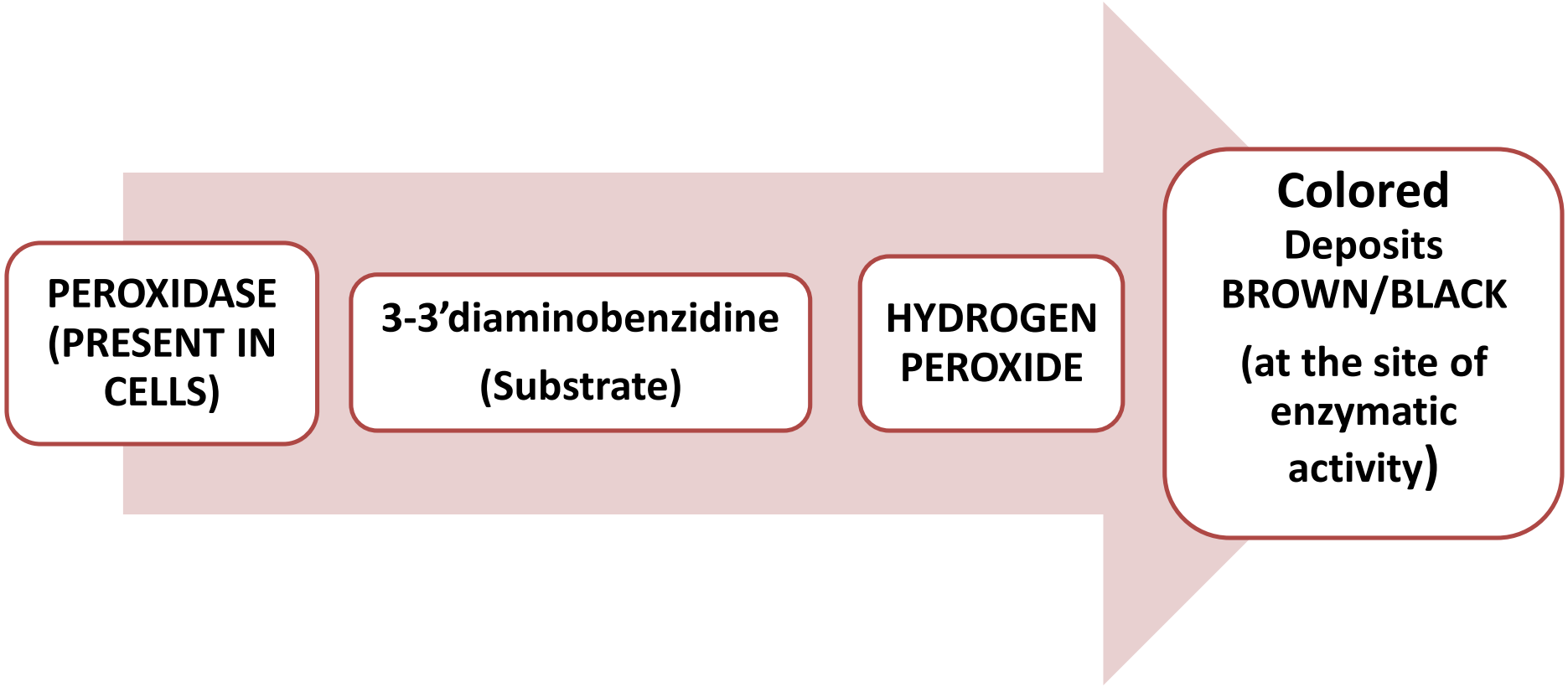
- Purpose
- Principle
- Reagents
- Quality
- Interpretation

MYELOPEROXIDASE (MPO)

Purpose

To look for the presence of myeloperoxidase enzyme in the blasts.

Principle



Fixative

95% Ethanol 45 ml + 40% Formaldehyde 5 ml.

Incubation Mixture

No	Ingradients	Quantity
1	30% Ethanol	100ml
2	Benzidine Dihydrochloride (Sigma B-3125)	0.3gm
3	0.132M Zinc Sulphate (3.8% W/V)	1.0ml
4	Sodiun Acetate Trihydate	1.0gm
5	3% Hydrogen Peroxide	0.7 ml
6	0.1N Sodium Hydroxide (4.0% W/V)	1.5ml

The final pH should be 6.00 +/- 0.05.

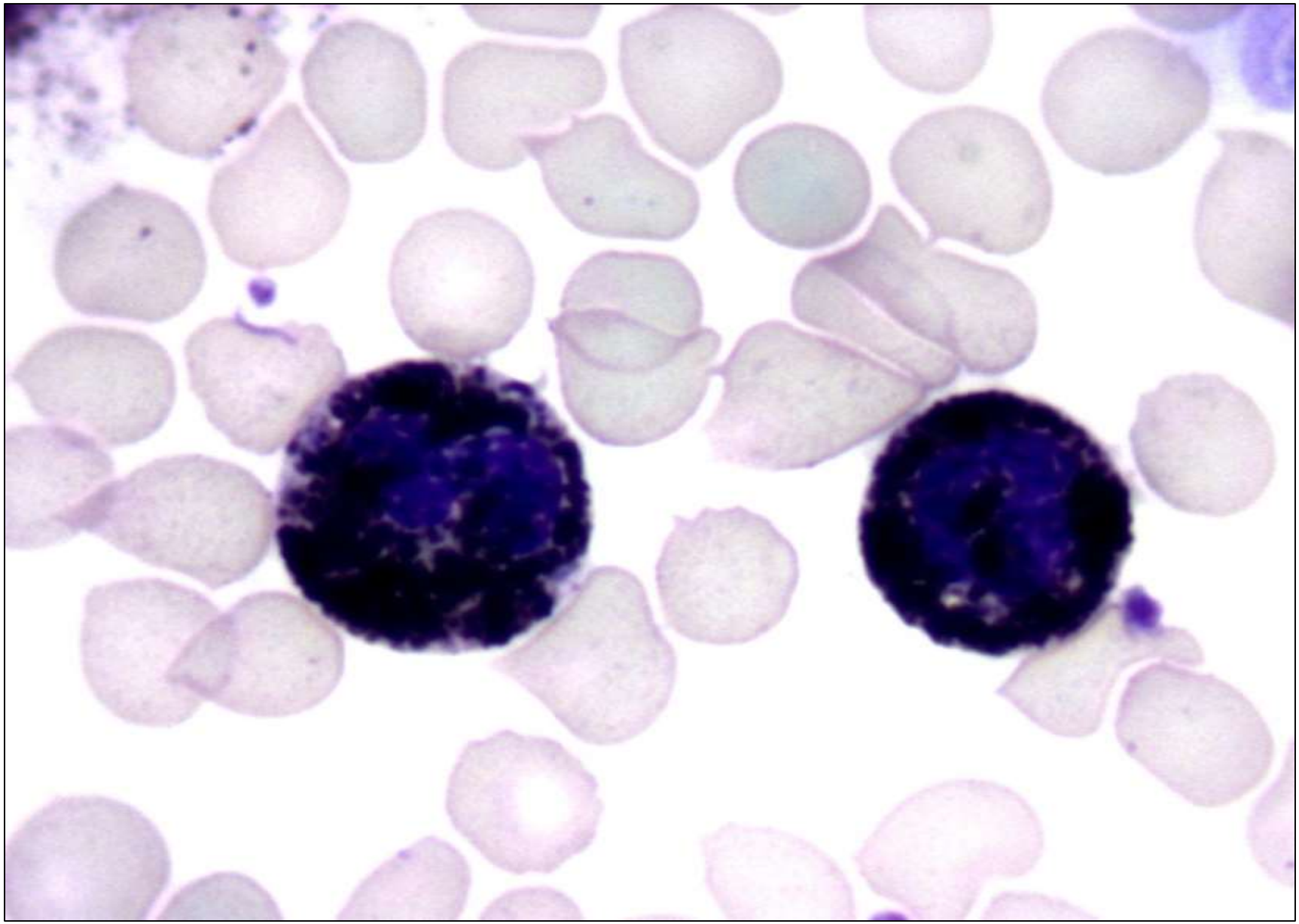
Quality control

Positive controls : **Myeloid series** cells (Neutrophil as internal contol)
Negative control: **Lymphocytes**

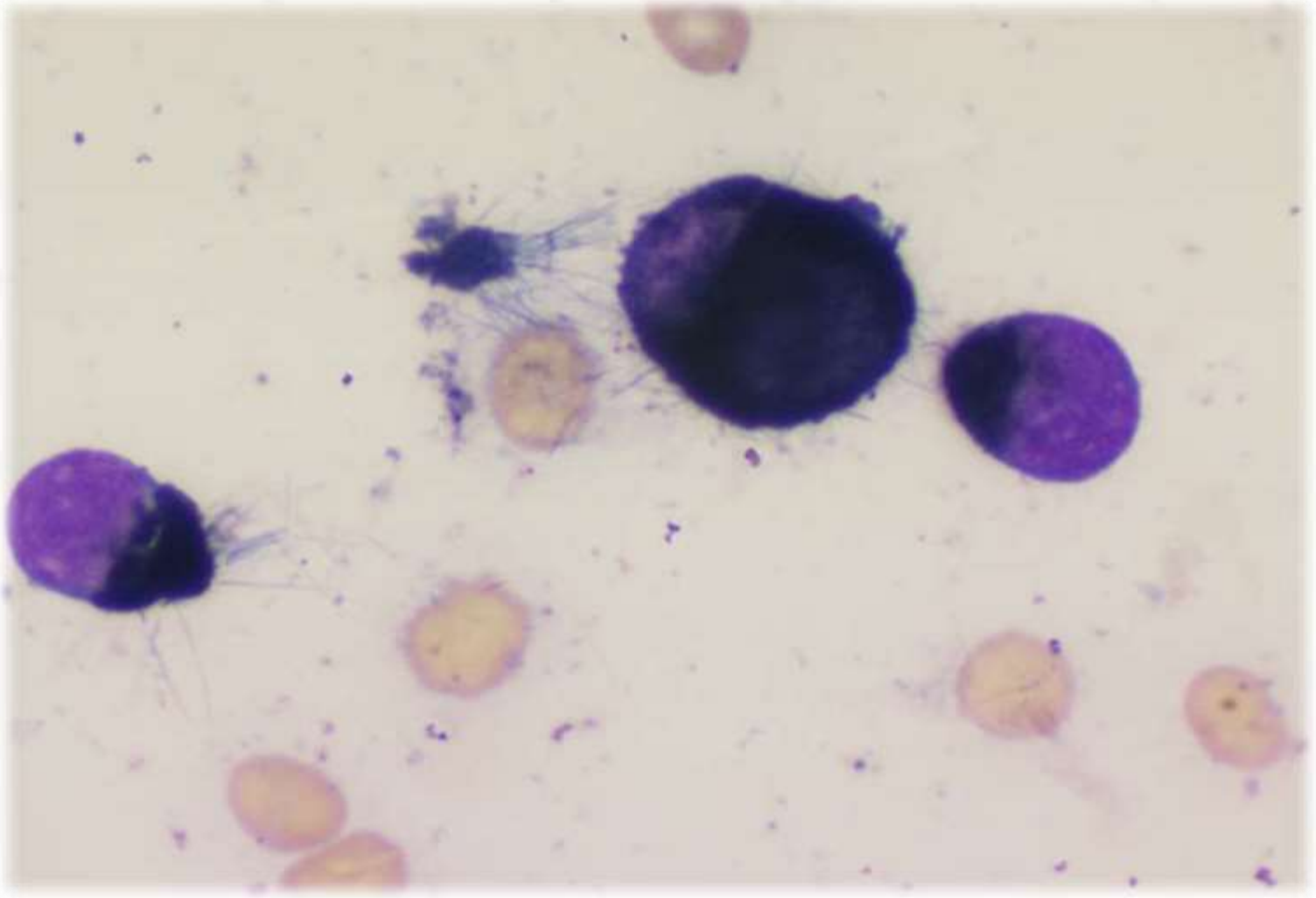
Interpretation

If >**3% blasts MPO positive**, the case is reported as **MPO positive.**
If **<3%** report as **negative.**

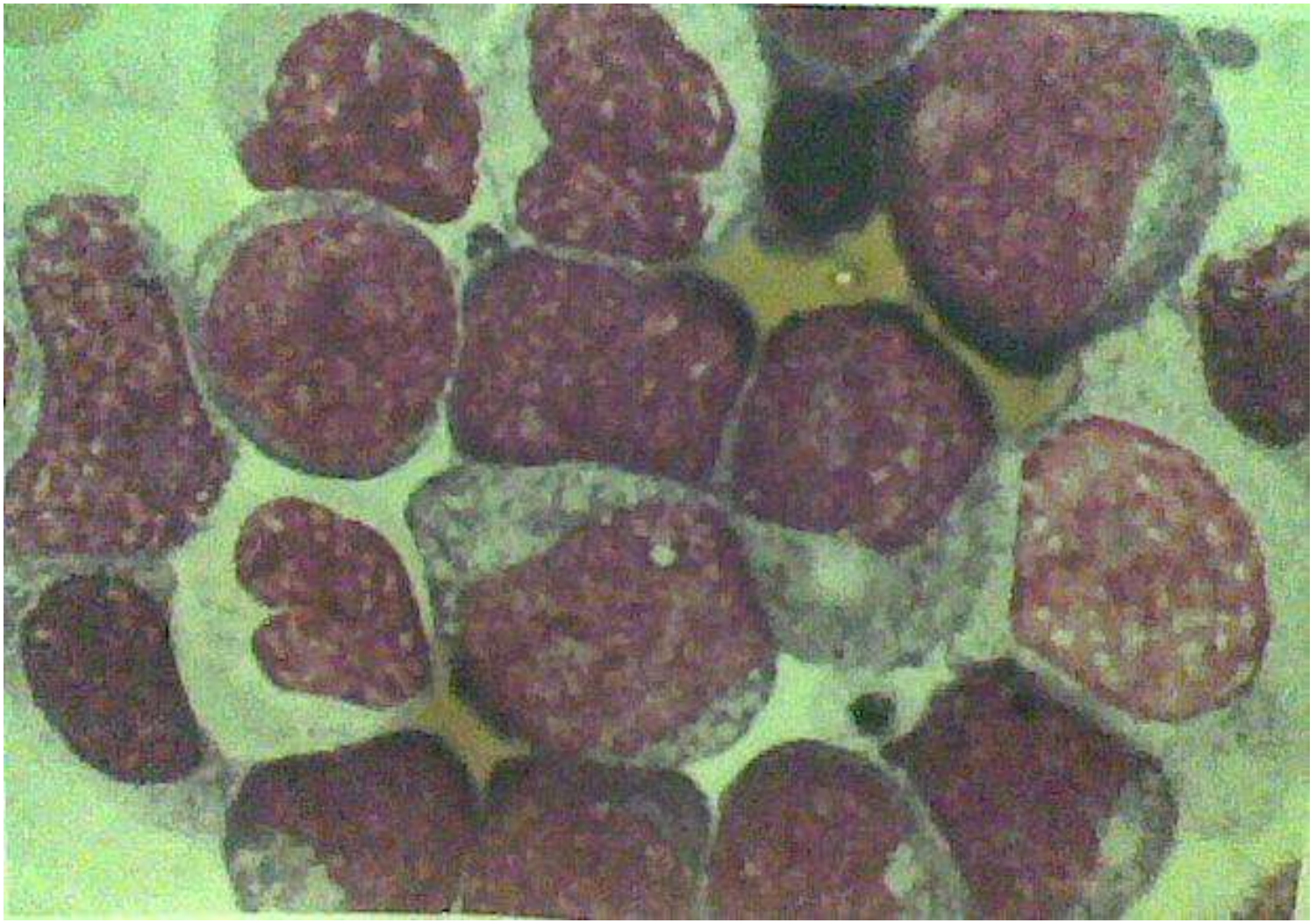
NOTE :If **internal control** (look in neutrophils and myeloid precursors) are **negative**, do not report and **repeat test**.



NEUTROPHILS SHOWING MPO POSITIVE



MPO POSITIVE BLAST



MPO NEGATIVE BLAST

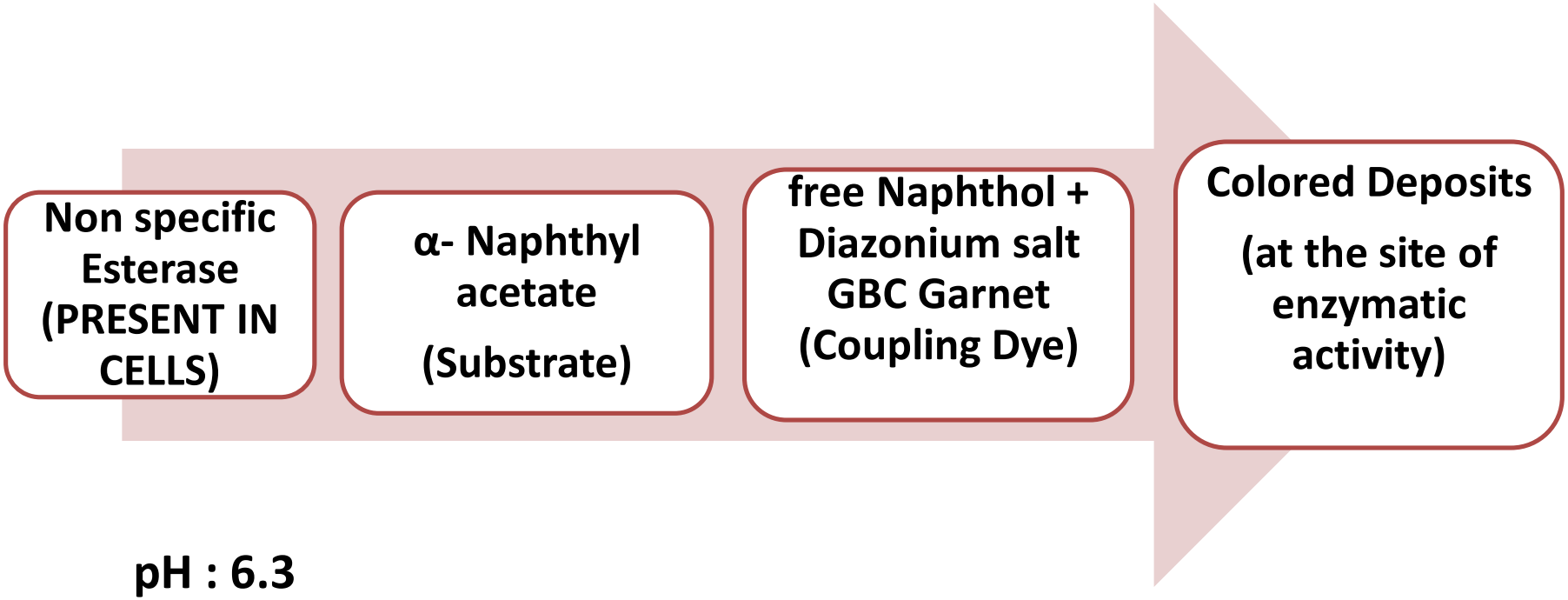
Non Specific Esterase (NSE)

- **Non- Specific Esterase** is used as one of the **diagnostic** criteria for **Acute Monocytic Leukemia (FAB** M5)

The cytochemical demonstration of esterase in leukocytes has chiefly been of practical use for **distinguishing granulocytes & their precursors from monocytes & monoblasts.**

PRINCIPLE

Leukocyte **esterases** are group of enzymes that **hydrolyze** acyl or chloroacyl **esters of α-napthol or napthol AS.**
The non-specific esterases are inhibited by sodium fluoride (NaF).



Reagents

1. **Fixative 40% Formaldehyde**

2.**Phosphate buffer** pH **6.3**

3. **Alpha naphthyl Acetate** (Sigma-8505)

4.Acetone (Excel R or E Merck)

5.**Fast Garnet GBC salt** (Sigma F 8761)

6.Sodium fluoride (NaF) (BDH)

7.**Aqueous Haematoxylin (COUNTER STAIN)**

Quality Control

- pH of the Phosphate buffer is checked every time on calibrated pH meter

- Known positive control is always used along with the test batch

- **Megakaryocytes and platelet series** show strong positivity and are taken as **internal positive control**

Interpretation

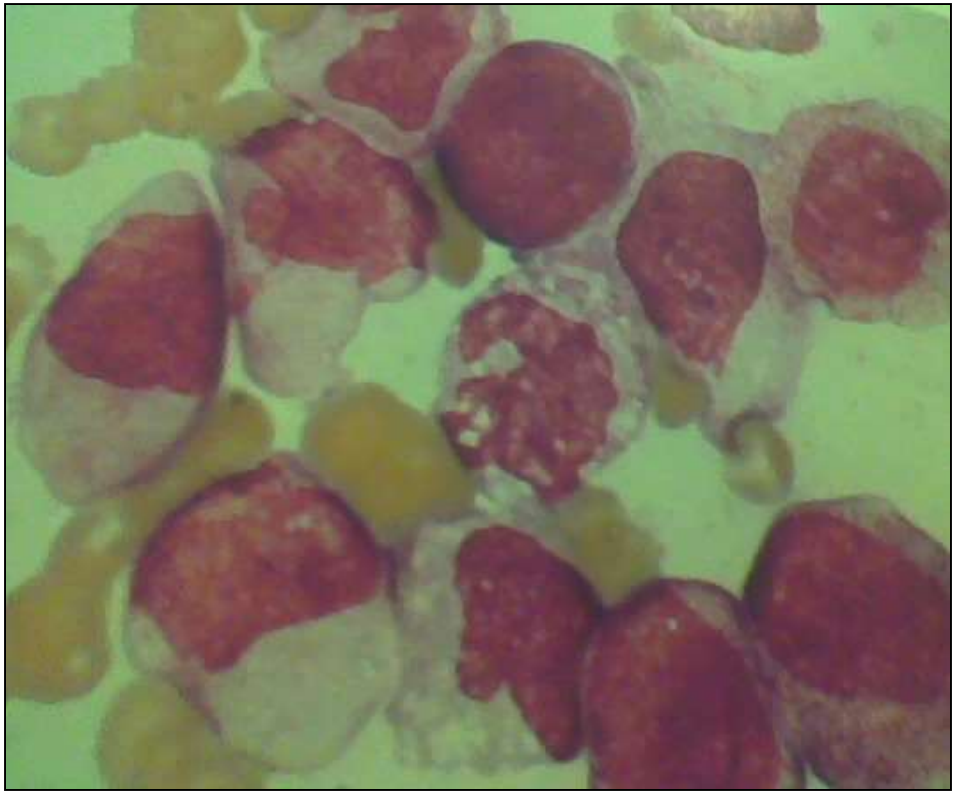
- The reaction product is **diffuse red /brown** in colour.

Positive reaction :

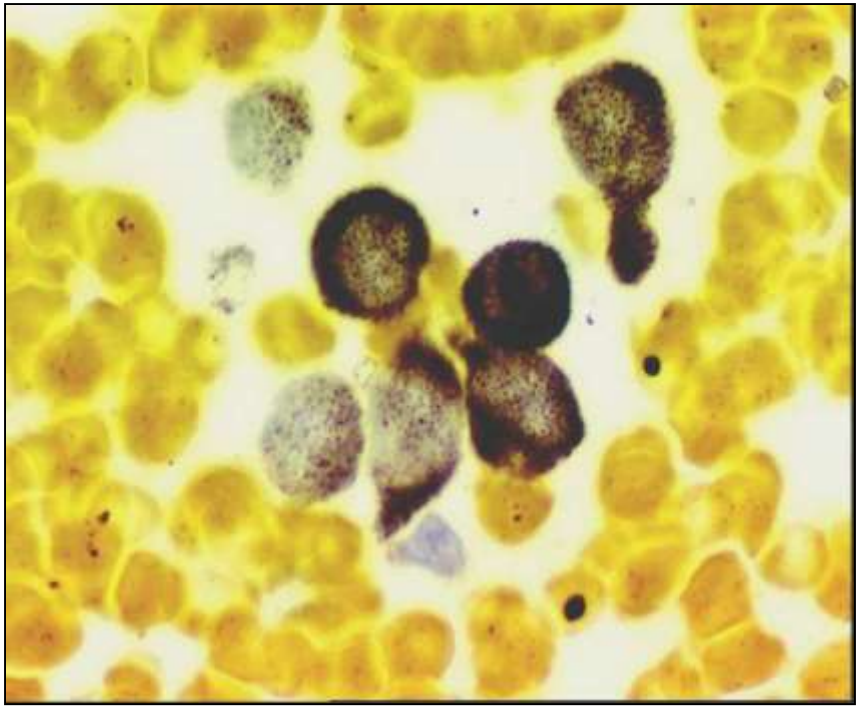
- Monocytes(normal as well as Blast)
- Megakarocytes (leukemic megakaryoblasts may show focal or diffuse positivity)
- **T lymphocytes** and some T lymphoblasts show focal **'dot like' positivity**.
- Leukemic **erythroblasts** may show **focal or diffuse** positivity

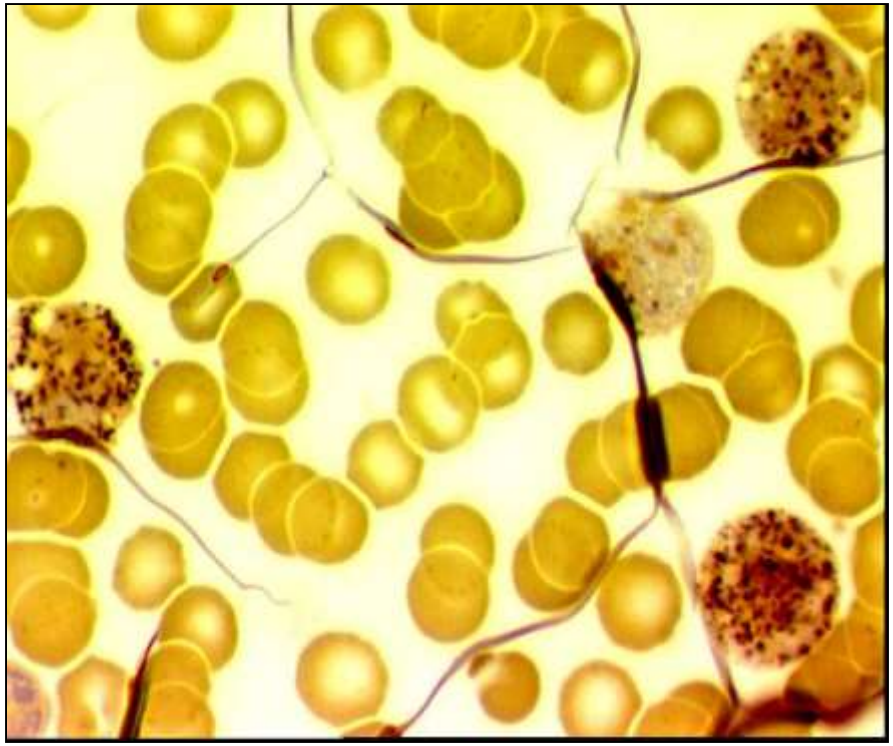
NEGATIVE REACTION :

Normal granulocytes are negative, but in myelodysplasia or AML may give positive reactions of varying intensity.



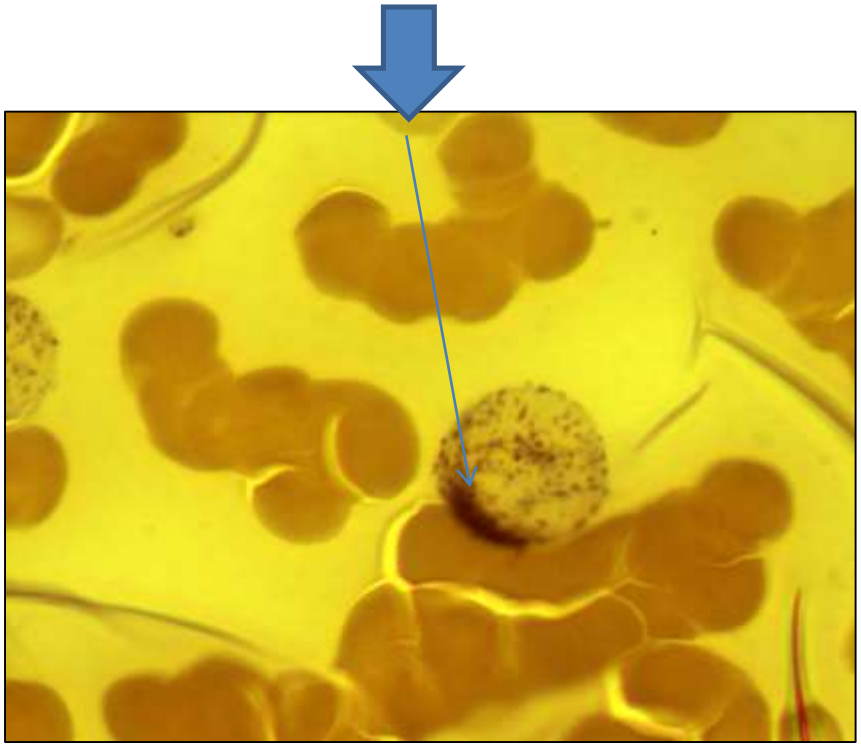
PS SHOWING Promonocytes and Monoblast





PS SHOWING NSE POSITIVE CELLS

NSE Positivity



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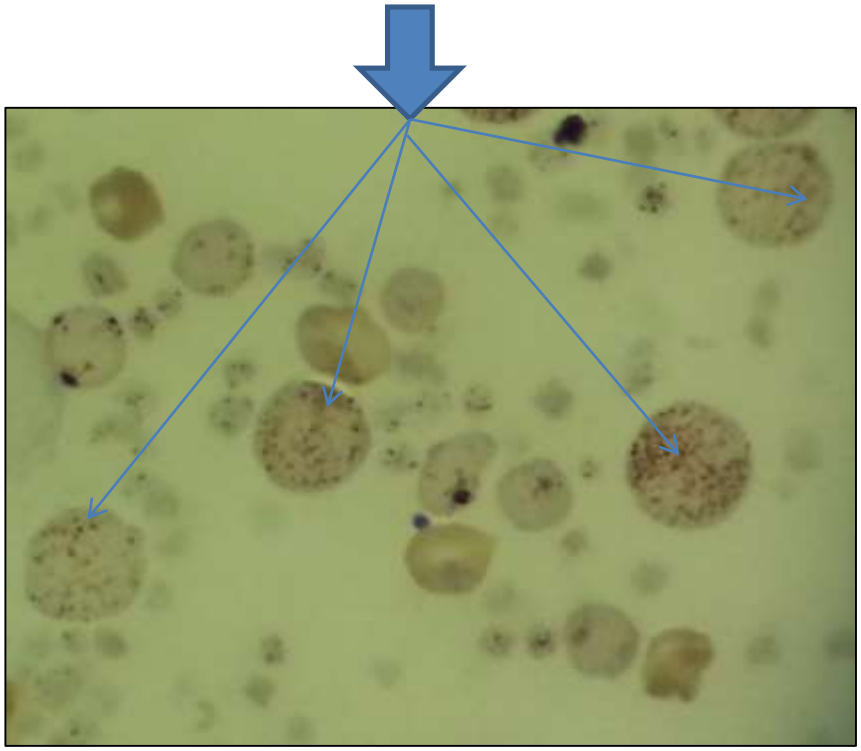

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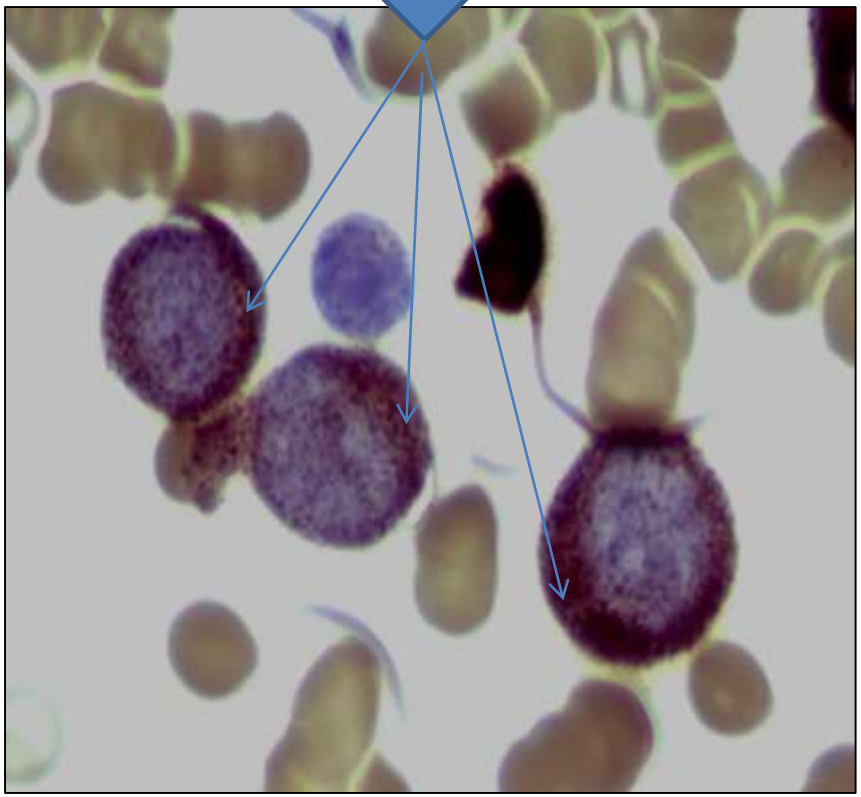

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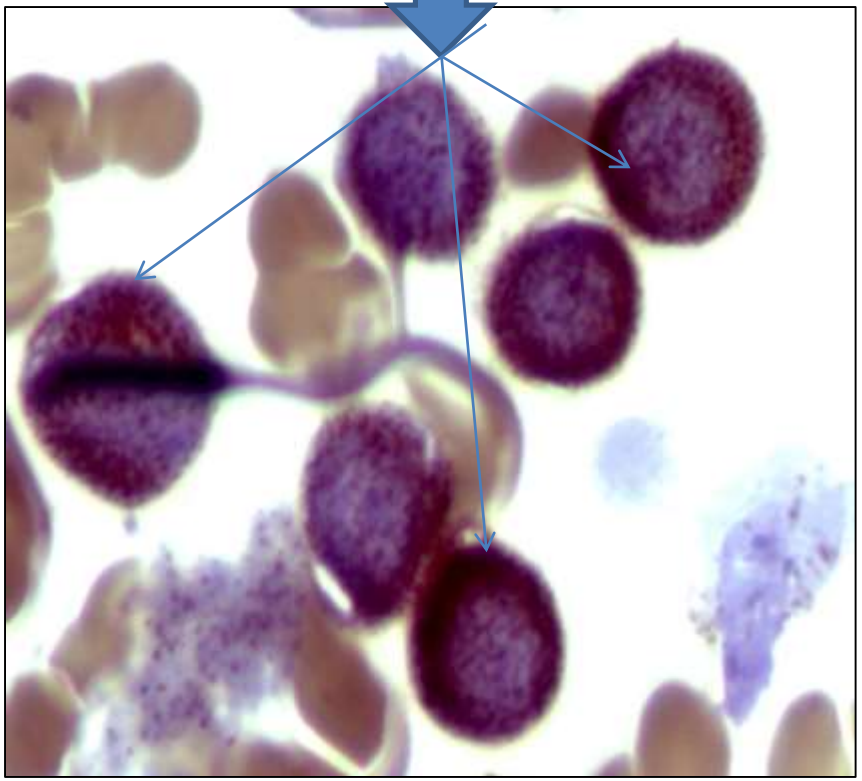
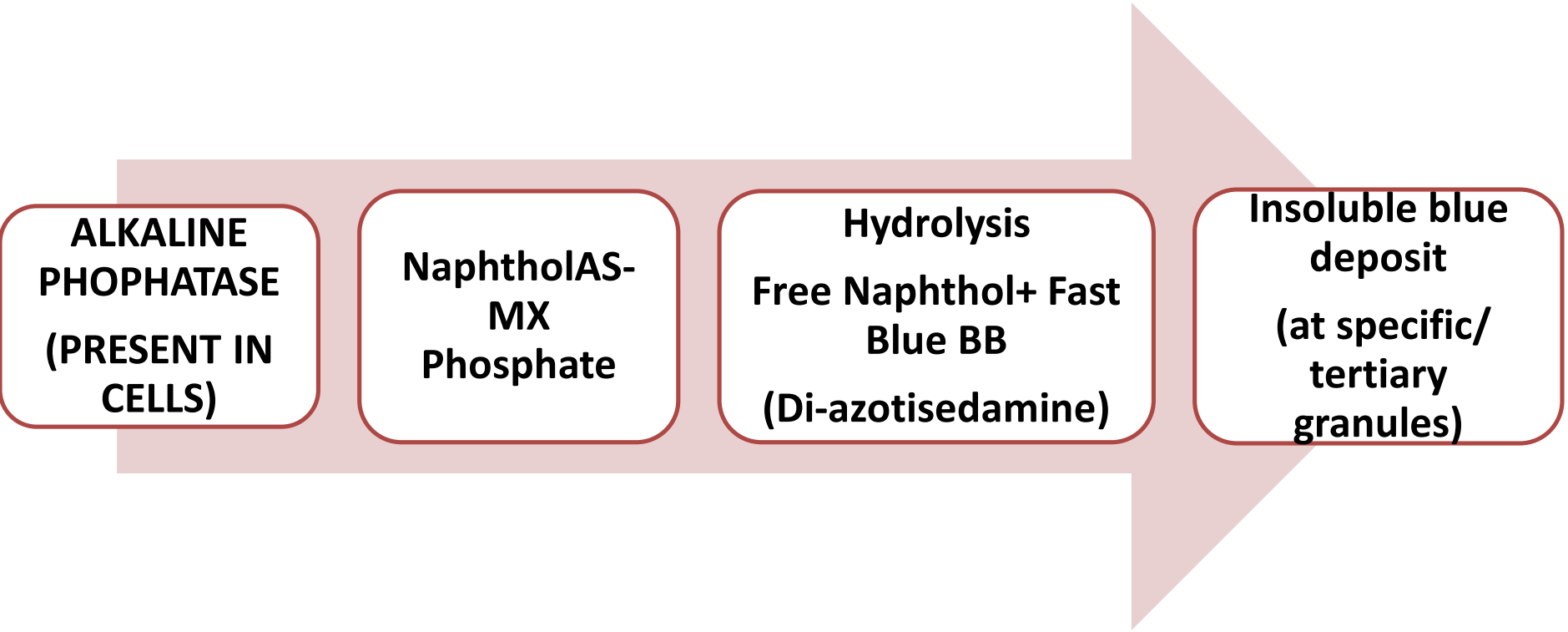


Leukocyte Alkaline Phosphatase (LAP)

Purpose

LAP is a **diagnostic criteria** for Chronic Myeloid Leukemia (**CML**).

PRINCIPLE



Reagents

1. Fixative 40% Formaldehyde 1ML +ABSOLUTE METHANOL 9ML
SHOULD BE kept at 20°C or in ice compartment of a refrigerator for up to 5-6 weeks.

2. TRIS BUFFER PH 9.0

3. Naphthol AS-MX Phosphate (sigma N-5000)

4. N N DIMETHYL FORMAMIDE (sigma D-4254)

5.Fast BLUE BB (DIAZONIUM SALT) (sigma F-4254) – (- 20°C)

6.NEUTRAL RED (COUNTER STAIN) (sigma N-7005)-1%aques neutral red

Calculation and Interpretation

- **The reaction product is blue and granular.**
- An overall score is obtained by assessing the stain intensity in **100 consecutive neutrophils**, with each neutrophil **scored** on a scale of **0–4** as follows
- **Normal Range:35 to 100**

Positive controls always give high results.

Interpretation based on granules	Score
Negative No granules	0
Occasional granules scattered in the cytoplasm	1
Moderate granules	2
Numerous granules	3
Heavily positive, Coarse granules overlying nucleus	4

Low score (< 35)

Chronic Myeloid Leukemia
Paroxysmal Nocturnal Haemoglobinuria (PNH)

High score (>35)

Neutrophilia of infection
Leukemoid reaction
Hepatic cirrhosis
Down's syndrome
Polycythemia Vera.

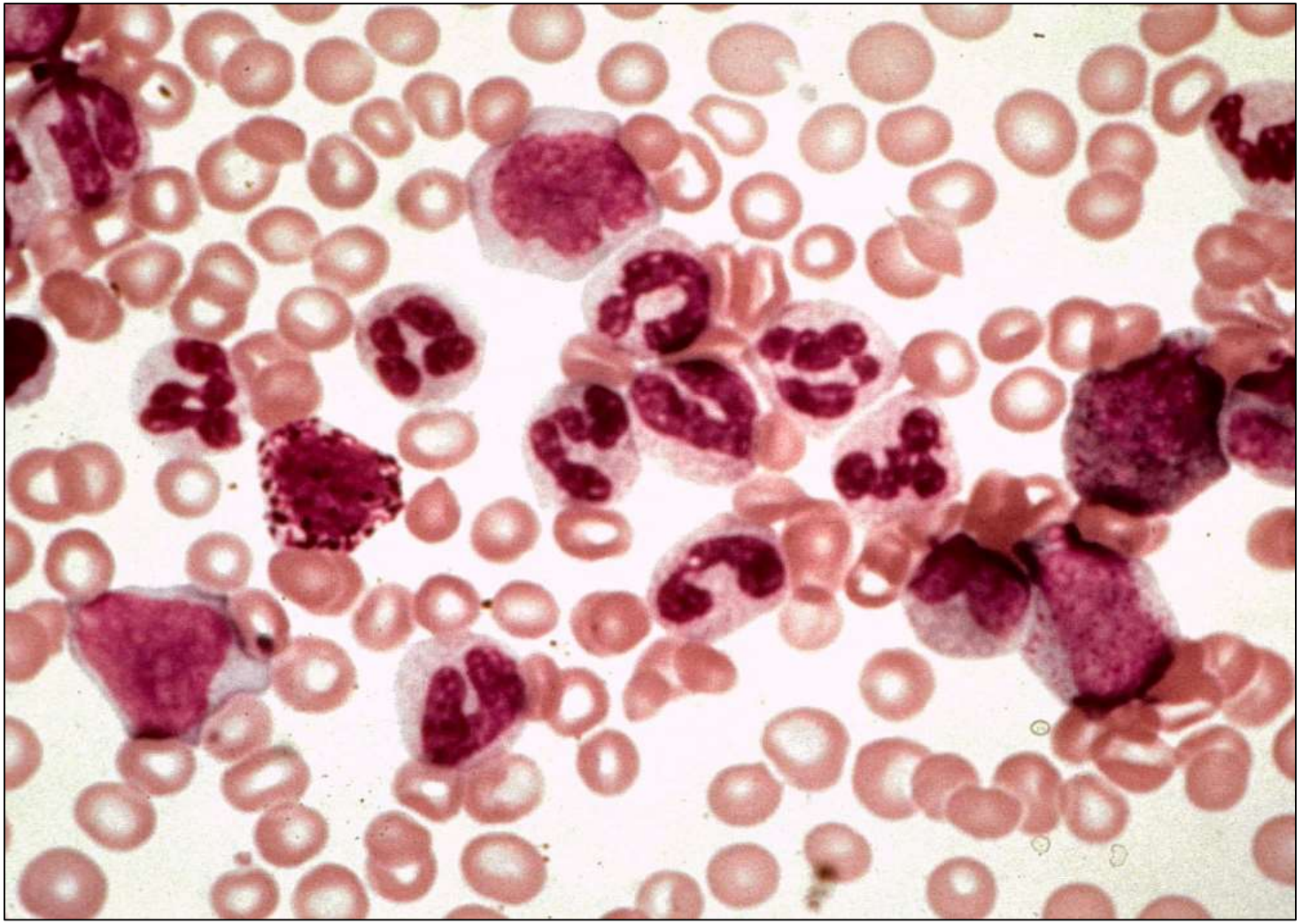
Quality Control

- **pH of the Phosphate buffer is checked every time on calibrated pH meter.**

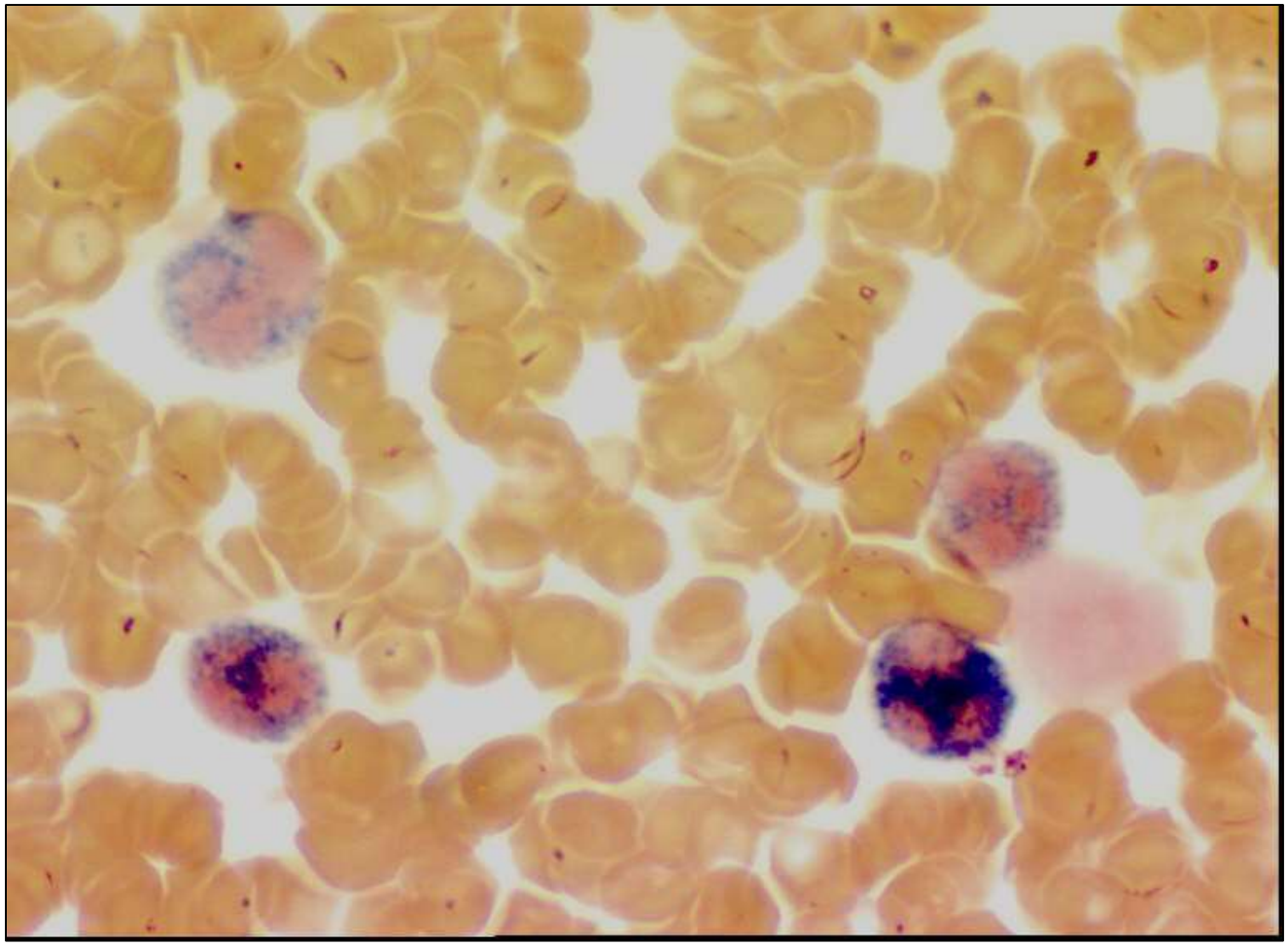
- Known positive control(**sample of pregnant women, infectious leukocytosis and other positive cases)** is always used along with the test batch.

- Anticoagulant like EDTA, heparin gives less satisfactory morphology.

- If anticoagulated blood is to be used, **smear should be made; as soon as possible and without any delay it should be fixed within two hour. Fixed slides can be stored for 3-4 days at 0°C or in a chilled tray by wrapping it in a tissue paper or aluminium foil**



Chronic Myeloid Leukemia



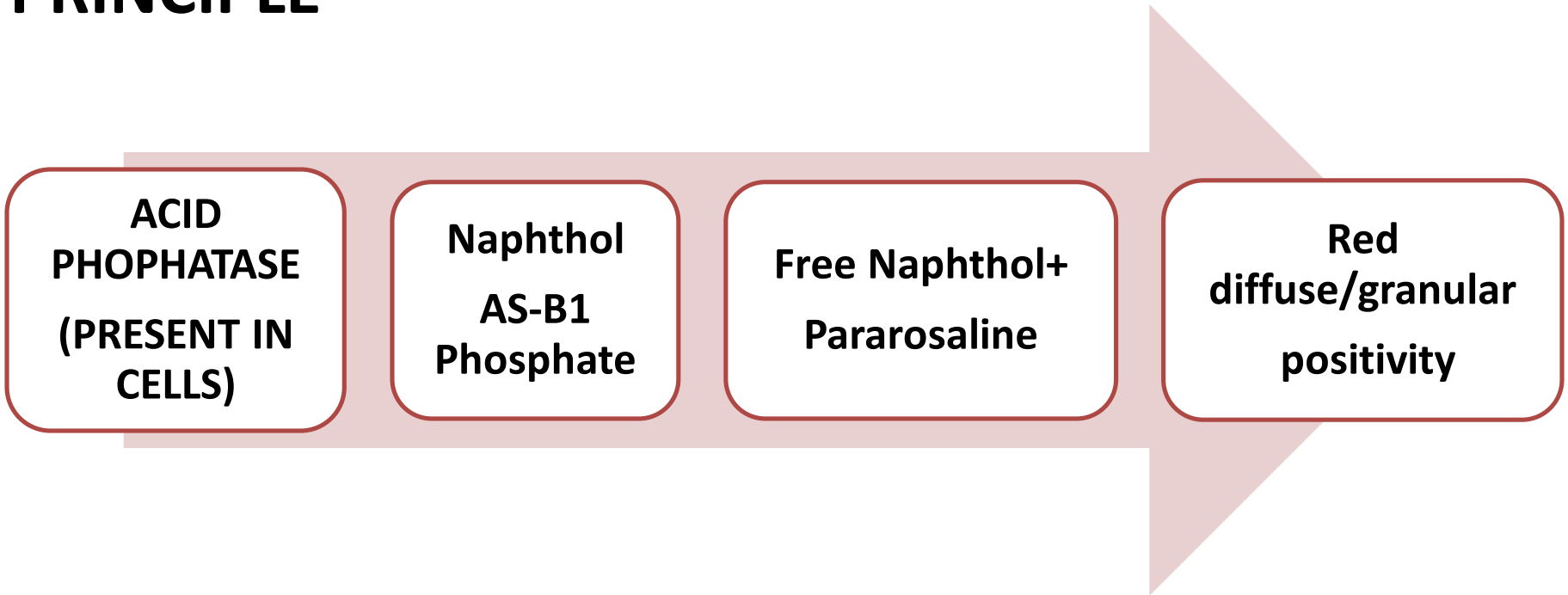
NEUTROHILS SHOWING LAP STAINING

TARTARATE RESISTANCE ACID PHOPHATASE

PURPOSE

➢Its main diagnostic use is in the **diagnosis of T-cell acute leukemias and hairy cell leukemia, but these diseases are more reliablely diagnosed and characteristized by immunophenotyping.**

PRINCIPLE



Reagents

1. **4% Sodium nitrite ($NaNO_2$) -** Always prepare fresh

2. **Pararosaline hydrochloride** (Sigma P -3750)

3. **Acetate buffer**

4. **2% Methyl green** (Sigma M-8884)

5. **Naphthol AS-B1** (Sigma N-2125) or **AS-MX** (Sigma N-4875)

6. **N-N-dimethyl formamide** (Sigma – 4254)

7. **Sodium tartarate**

Quality control

- Monitor the pH of the acetate buffer (pH 5.2)

- Each batch of analysis should have **a positive control** . Either known control or the slide from patient of **T cell ALL** or the slides showing **T reactive lymphocytes** (localized cytoplasmic positivity) **or plasma cell leukemia** or **myeloma** can be taken.

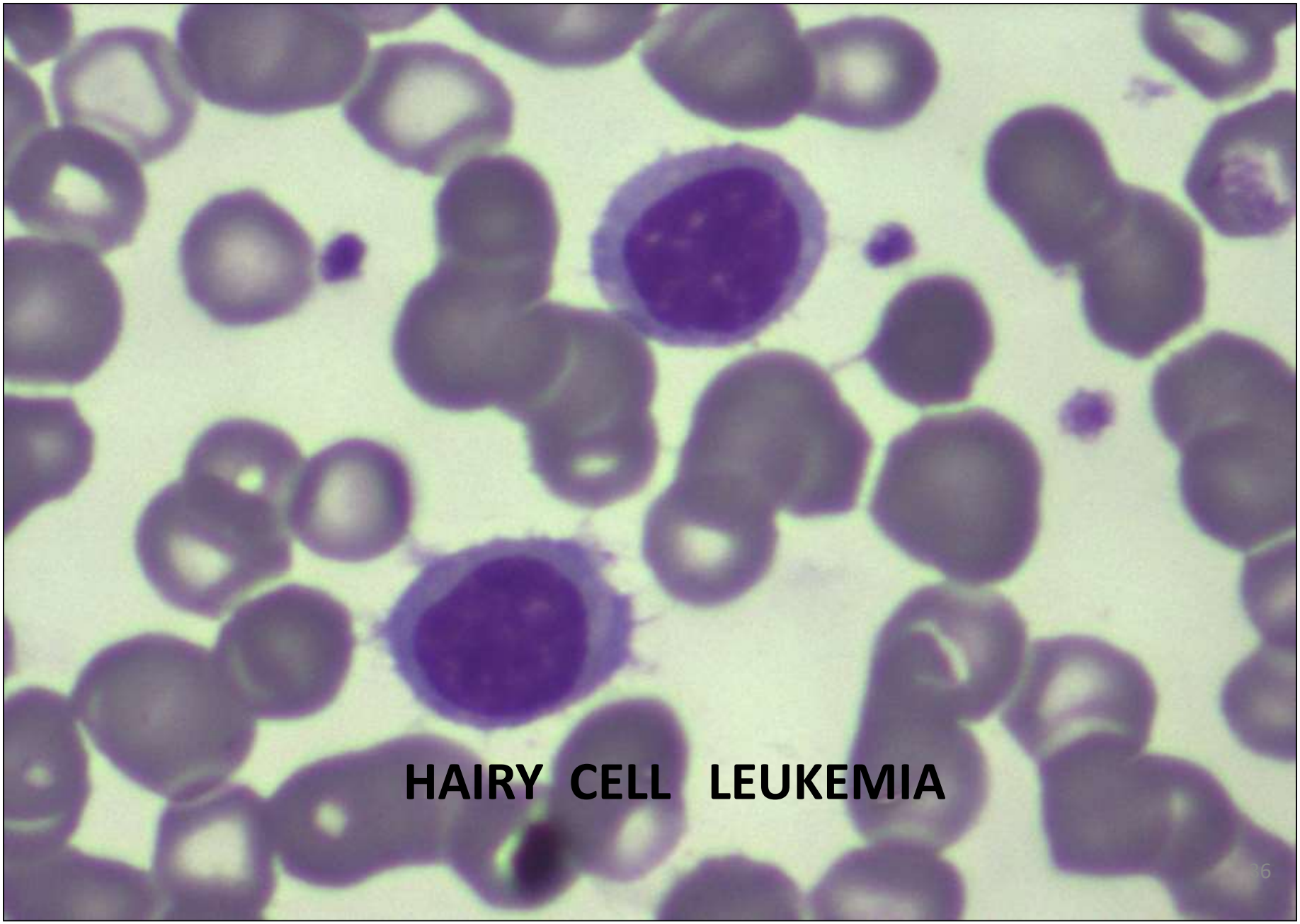
- **Granulocytes** are positive, hence can be taken as **positive control.**

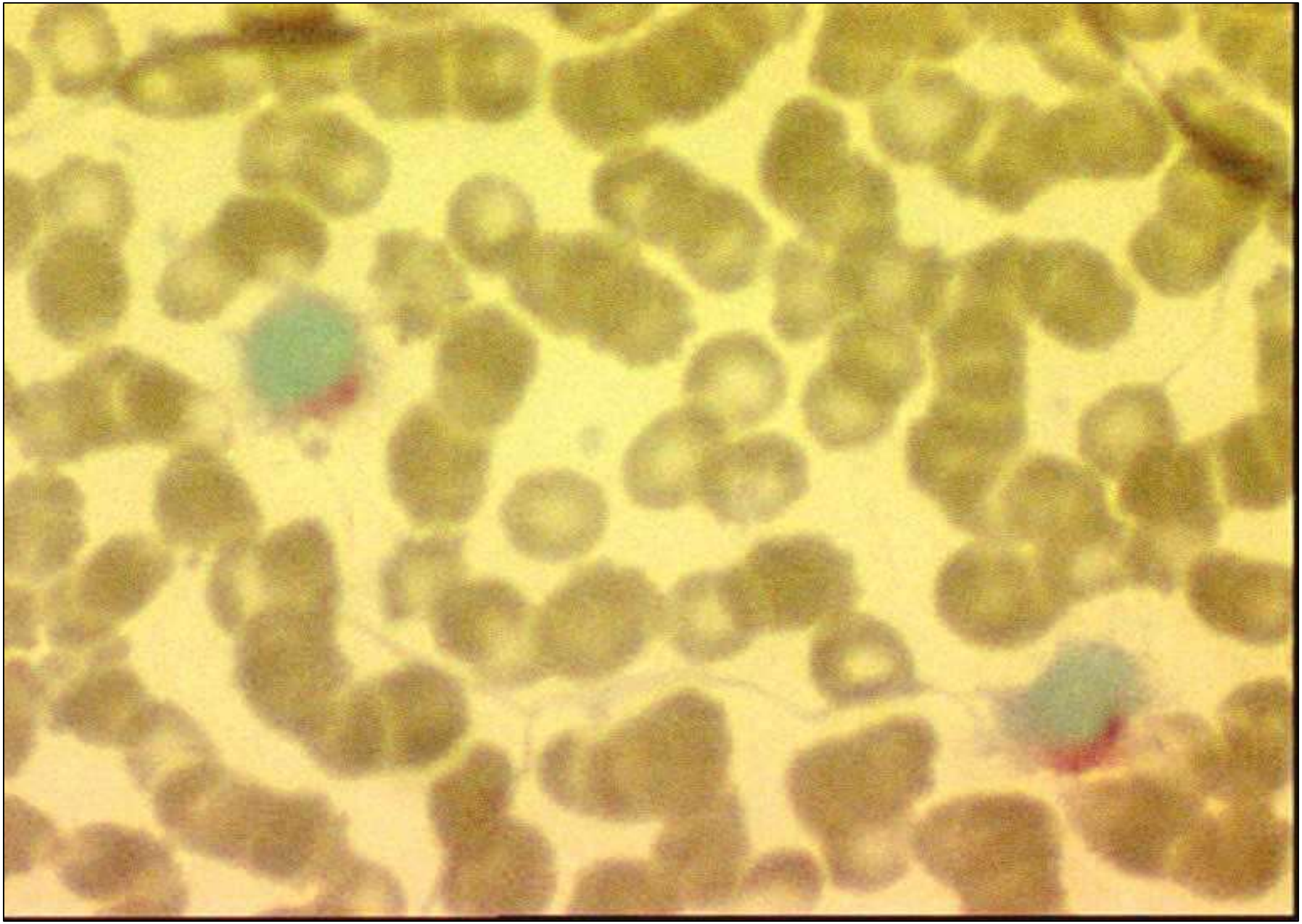
Interpretation

- Its main diagnostic use is in the **diagnosis of Hairy cell leukemia**, which are resistant to tartaric acid.

- The reaction product is **red** with a mixture of **granular positivity.**

- In **T cell**, acid phosphatase is usually **positive showing localized activity (polar).**


HAIRY CELL LEUKEMIA



TARTRATE RESISTANCE HAIRY CELLS

Iron Staining/ Perl's reaction/ Prussian Blue staining

Purpose

To measure the iron stores and for evaluation of ring –sideroblast on bone marrow smears.

Principle

Siderocytes are red cells containing granules of non-haem iron. The granules are formed by water-insoluble complex of ferric iron, lipid, protein & carbohydrate.

This **siderotic material** (or haemosiderin) reacts with **potassium ferrocyanide** to form a blue compound, **ferriferrocyanide**, this reaction is the basis of a positive **Prussian – blue** (**Perl's**) reaction.

Reagents

1. Absolute methanol (Fixative)

2. 2% Potassium ferrocynide
(2 gms dissolved in 100 ml D/W) Keep this
solution in brown bottle. This solution is stable for 2 months.

3. 2% HCl

4. 0.1% Aqueous Eosin solution or 0.1% Neutral red

5.Prussian blue reagent
Prepare solution immediately before use by mixing equal volume of 2% potassium ferrocynide & 2% HCl

Quality control

- **A positive control is always kept by taking bone marrow slide of patient with Erythroid hyperplasia, Hodgkin disease or Non -Hodgkin lymphoma.**

- **BM slide should be selectecd in which particulate matter is present.**

Interpretation

- Look for the ring sideroblasts on the smear.

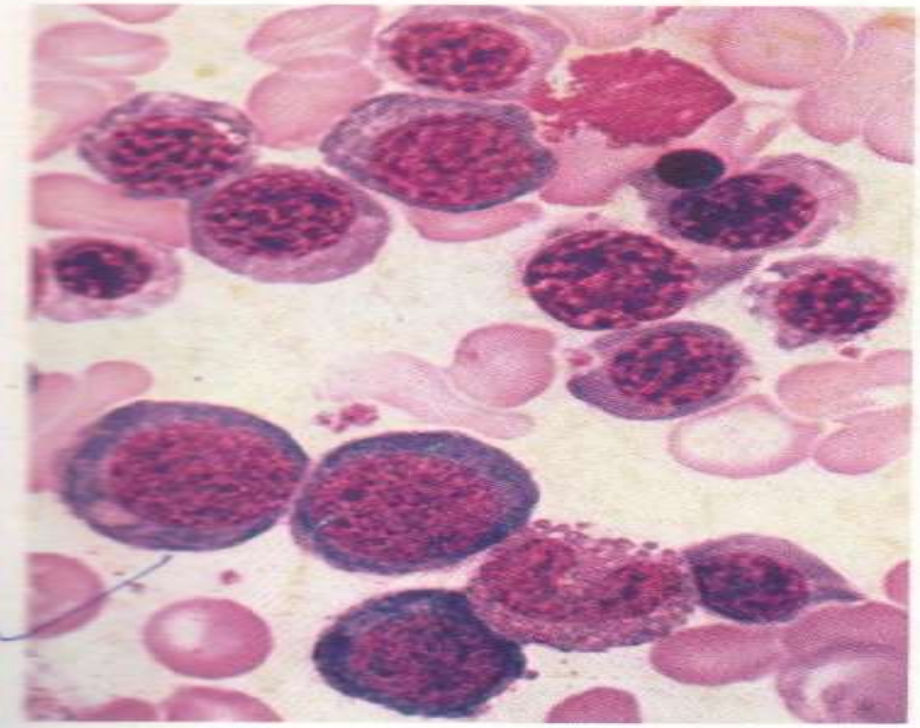
- Results are reported as adequate, moderate or deficient depending on the amount of intensity and amount of iron present.

- Iron store Reduced or absent in iron -deficiency anemia.

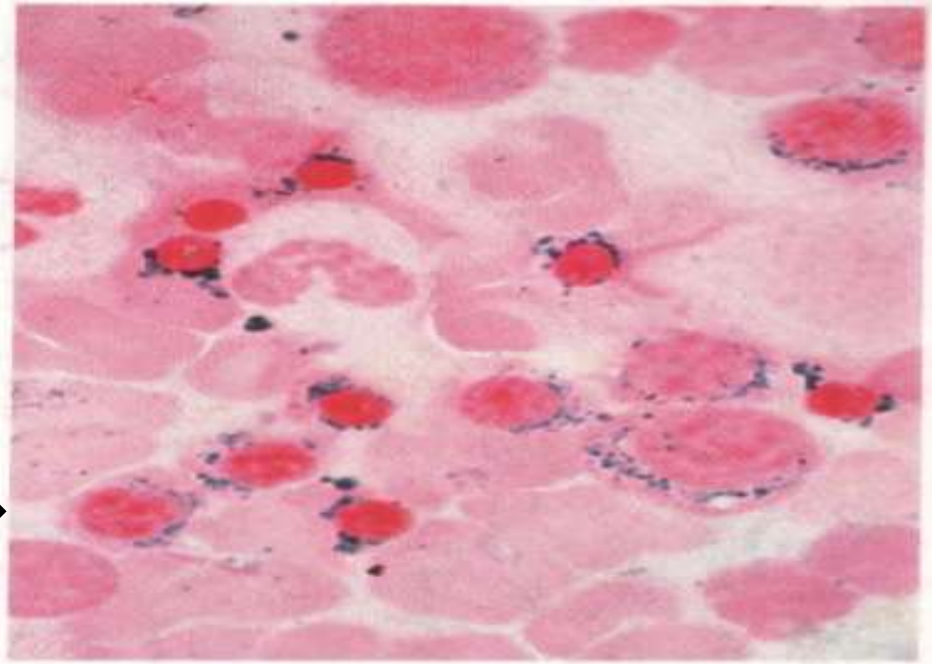
- Increased when there is iron overload , in dyserythropoietic anaemias, sideroblastic anaemias and thalassemia.

- Sideroblastic anaemias shows Perinuclear ring

- **Ring sideroblasts seen in Refractory anemia ,MDS**



Erythroid hyperplasia in bone marrow aspirate smear



Perl's stain demonstrating Ring sideroblasts

References :

- Practical Haematology, 9th Edition, Dacie & Lewis
- Haematological Cytochemistry, F.G.J.Hayhoe, Kaplow L S
- ICSH reference method for staining of blood and bone marrow films by azure B and eosin Y (Romanowsky stain). International Committee for Standardization in Haematology. Br J Haematol. 1984 Aug;57(4):707-10

THANKS