Cytodiagnosis is the diagnosis of disease through the microscopic examination of cells (of human, animal or plant origin) collected by various means.

In the case of human cytodiagnosis, there are two areas of cytology – gynecological and non-gynecological – in which specimen material is examined for the presence of malignant and premalignant cells, which, through certain procedures, may be classified as:

- Normal
- Inflammatory
- Suspect / Uncertain
- Malignant
WHAT IS “PAP TEST”?

• Pap test is a method of examining with a microscope a sample of superficial cells that line the inner wall of the uterine cervix to detect any abnormal cell for early diagnosis of uterine cancer.

• It was developed in 1928 by the Greek doctor George Nicholas Papanicolaou (1883-1962) at the Cornell Medical College of New York. He also developed the particular polychrome staining reaction designed to demonstrate variations of cellular maturity and metabolic activity.

• The name “Pap test" derives from the first letters of his surname.
The Papanicolaou stain is also used for non-gynecological (clinical) material. For instance, specimens of sputum or urine, containing squamous epithelial or similar cells, demonstrate excellent results when stained according to the Papanicolaou technique.
Papanicolaou stain

Hematoxylin stain  >  Orange stain  >  Polychromic stain

1. Result
2. Result
3. Result

Hematoxylin stain  +  Orange stain  +  Polychromic stain  =  Papanicolaou stain
Papanicolaou stain

The three main advantages of this staining procedure are:

1. Good definition of nuclear detail.
2. Cytoplasmic transparency.

It is a polychrome staining method which depends on degree of cell maturity and cellular metabolic activity. Cytoplasmic transparency is a function of high ethanol concentration of the stain. This is important in order to view multilayered cell aggregates.
PRINCIPLES OF PAP STAIN

• HAEMATOXILYN (violet): a basic stain with chemical affinity for acid substances (i.e. nuclei of cells, filled with DNA).

• EA50 (light blue): an acid stain that reacts with the cytoplasm of less mature squamous (exocervical) cell (basal, parabasal and intermediate cell) and with glandular (endocervical) cell.

• OG6 (orange): an acid stain that reacts with superficial squamous cell, filled with keratin.
The haematoxylin nuclear stain demonstrates chromatinic patterns of normal and abnormal cells.

The counterstains, Orange-G and E.A. (eosin-azure) have a high alcoholic concentration which provides cytoplasmic transparency. This enables clear visualization through areas of overlapping cells, mucus and debris.

There are four main steps in the staining procedure:

1. Fixation.
2. Nuclear staining.
3. Cytoplasmic staining.
# Staining steps

1) **Ethanol 95° (Fixation)**  
   2 minutes

2) **Distilled water**  
   2 minutes

3) **Harris Hematoxylin**  
   1 minute

4) **Tap water**  
   5 minutes

5) **Ethanol 95°**  
   15 seconds

6) **OG 6**  
   2 minutes

7) **Ethanol 95°**  
   15 seconds (twice)

8) **EA 50**  
   5 minutes

9) **Ethanol 95°**  
   15 seconds

10) **Absolute Ethanol**  
    30 seconds (twice)

11) **Xilene or Bio Clear**  
    2 minutes (twice)
Fixation

• **Specimens must be fixed immediately after being taken and while still moist!!!!**
  • To prevent drying out and shrinking of cells
  • To maintain specimen’s structural features
  • To permit clear staining and differentiation

• If specimens are fixed too late, so-called **artifacts** can be found in Papanicolaou-stained smears on single cells or cell clusters.

• The classic method of fixing is to immerse the microscope slides in 96% ethanol for 30 min.

• A more efficient and quicker way is to fix them with a spray fixative. Spray fixatives are aqueous-alcoholic solutions containing polyethylene glycol (PEG, Carbowax), and are suitable for all types of cytological material due to be stained by the Papanicolaou method.
Air drying artifacts

Air drying is a physiochemical process where there is more or less complete loss of water from the cells (especially from the nuclei) connected with structural alterations of the cell, as spreading of the nucleus or reduction of the staining reaction after application of the PAP stain.

Alterations caused by airdrying:
1. Spreading of cells on the slide surface with a change of nuclear area. 3D cell nuclei become flat,
2. Condensation of chromatin. This cannot be fully restored after reimmersion in water,
3. Favouring/preventing staining reactions.
## Staining steps

1. Ethanol 95° (Fixation)  
2. Distilled water  
3. Harris Hematoxylin  
4. Tap water  
5. Ethanol 95°  
6. OG 6  
7. Ethanol 95°  
8. EA 50  
9. Ethanol 95°  
10. Absolute Ethanol  
11. Xilene or Bio Clear
• From fixative (95% alcohol) the cells are hydrated through a graded series of alcohols to water preparatory to haematoxylin immersion (the haematoxylin is an aqueous solution).

• The cells are then dehydrated prior to immersion in the alcohol based cytoplasmic counterstains.

• Grading the alcoholic solutions in a stepwise manner is thought to minimise cellular distortion and reduce cell loss from the glass slide, due to convection currents in the solutions.
Hematoxylin

✓ The haematoxylin nuclear stain is a natural stain which has been used for over 100 years in histology.

✓ It has affinity for chromatin, attaching to sulphate groups on the DNA molecule.

✓ Rinse under tap water to remove excess dye
Staining steps

1) Ethanol 95° (Fixation) 2 minutes
2) Distilled water 2 minutes
3) Harris Hematoxylin 1 minute
4) Tap water 5 minutes
5) Ethanol 95° 15 seconds
6) OG 6 2 minutes
7) Ethanol 95° 15 seconds (twice)
8) EA 50 5 minutes
9) Ethanol 95° 15 seconds
10) Absolute Ethanol 30 seconds (twice)
11) Xilene or Bio Clear 2 minutes (twice)
Orange G

• A monochromatic stain which colours keratin a brilliant orange.

• The effects of Orange G are only evident in smear when keratinised cells are present. However it is likely that it enhances red blood cell staining.
Staining steps

1) Ethanol 95° (Fixation) 2 minutes
2) Distilled water 2 minutes
3) Harris Hematoxylin 1 minute
4) Tap water 5 minutes
5) Ethanol 95° 15 seconds
6) OG 6 2 minutes
7) Ethanol 95° 15 seconds (twice)
8) EA 50 5 minutes
9) Ethanol 95° 15 seconds
10) Absolute Ethanol 30 seconds (twice)
11) Xilene or Bio Clear 2 minutes (twice)
EA-50 (Eosin – Azure)

- a polychromatic mixture of:
  1. Eosin G
  2. Light Green SF
  3. Bismarck Brown

- Various EA modifications are known. They differ simply through the various concentrations of the individual dyes.

- Staining solutions commonly used in cytology are EA 31 and EA 50, while EA 65 is preferred for mucous material such as sputum, bronchial secretions and other non-gynecological material.

- Bismarck Brown reportedly does not have a staining effect but rather contributes to stabilizing the staining solution.
• The two dyes Eosin G and Light Green SF compete for the same target structures and cause the cells to be differently stained at various cyclic stages.

• Mature squamous epithelial cells, nucleoli and ciliae, for instance, have a stronger affinity for Eosin G, while parabasal and intermediate cells appear green, blue-green or blue after being stained with Light Green SF.

**Omission of Orange G did not affect the accuracy of diagnosis (since keratin and red blood cell are also stained by eosin).**
## Staining steps

1) Ethanol 95° (Fixation)  →  2 minutes
2) Distilled water  →  2 minutes
3) Harris Hematoxylin  →  1 minute
4) Tap water  →  5 minutes
5) Ethanol 95°  →  15 seconds
6) OG 6  →  2 minutes
7) Ethanol 95°  →  15 seconds (twice)
8) EA 50  →  5 minutes
9) Ethanol 95°  →  15 seconds
10) Absolute Ethanol  →  30 seconds (twice)
11) Xilene or Bio Clear  →  2 minutes (twice)
Clearing

- Clearing in xylol results in cellular transparency and precedes mounting.
- **Xylol** is the commonest clearing agent and is miscible with alcohol (absolute only).
- Xylol is colorless, chemically non-reactive and has almost the same refractive index as glass which is important to give the best possible transparency of the image.
- The presence of water in xylol causes cloudiness due to water droplets. Water and xylol are immiscible.
Mounting

The mountant:
(a) acts as a permanent bond between slide and coverslip
(b) protects cell material from air drying and shrinkage
(c) acts as a seal against oxidation and fading of the stain.
Causes of inconsistent staining

1. varying thickness of material on slide
2. type of fixative used
3. inadequate filtering of stain solutions
4. age of staining solution
5. degree of usage of staining solutions
6. use of chlorinated tap water
7. pH of water can effect nuclear staining
8. temperature of water
9. insufficient rinsing after acid
10. air drying of slides between solutions
11. improper draining of slides during staining.
This common brown artifact is said to be caused by air bubbles formed when xylol dries before the slide is mounted. It can sometimes be so extensive as to render the slide unsuitable for evaluation. Remounting the slide can sometimes improve the appearance of the smear.