



Biological Screening Workshop

Blood

What Is Blood?

- **Slightly alkaline fluid made up of water, cells, enzymes, proteins, glucose, hormones, organic and inorganic substances**
- **Circulates throughout body**
 - **Supplies nutrients and oxygen to body**
 - **Removes waste**

Blood Cells

- **Cells mature and differentiate into several classes of cells:**
 - **Red blood cells**
 - **White blood cells**
 - **Platelets**

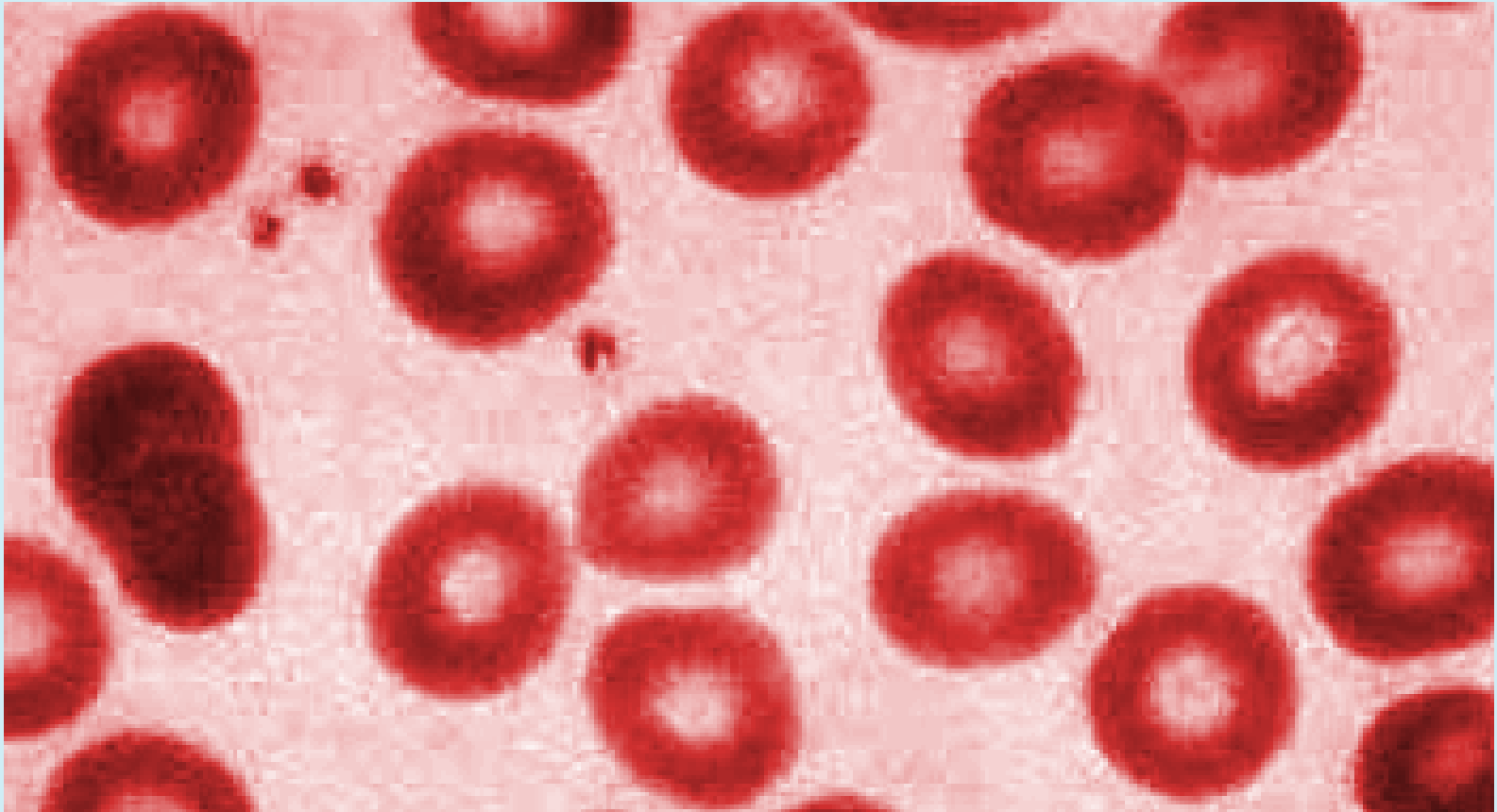
Red Blood Cells (Erythrocytes)

- **Have no nucleus**
 - **Not useful for DNA analysis**
- **Six to eight μm in size**
- **About 45% total volume of blood**
- **Most abundant cell in the blood**

Red Blood Cells

- **Disk shaped cells which make up 99% of the cells in the blood**
- **Principal carriers of the red colored hemoglobin molecules**
 - **Hemoglobin is an iron-containing protein and binds about 97% of all oxygen in the body**

Red Blood Cells



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White Blood Cells (WBC) (Leukocytes)

- **Produced in bone marrow**
- **WBCs have a nucleus**
 - **Useful for DNA analysis**
- **Vital source of defense against external organisms**
- **White blood cells also clean up dead cells and tissue debris that would otherwise accumulate and lead to problems**

Red and White Blood Cells

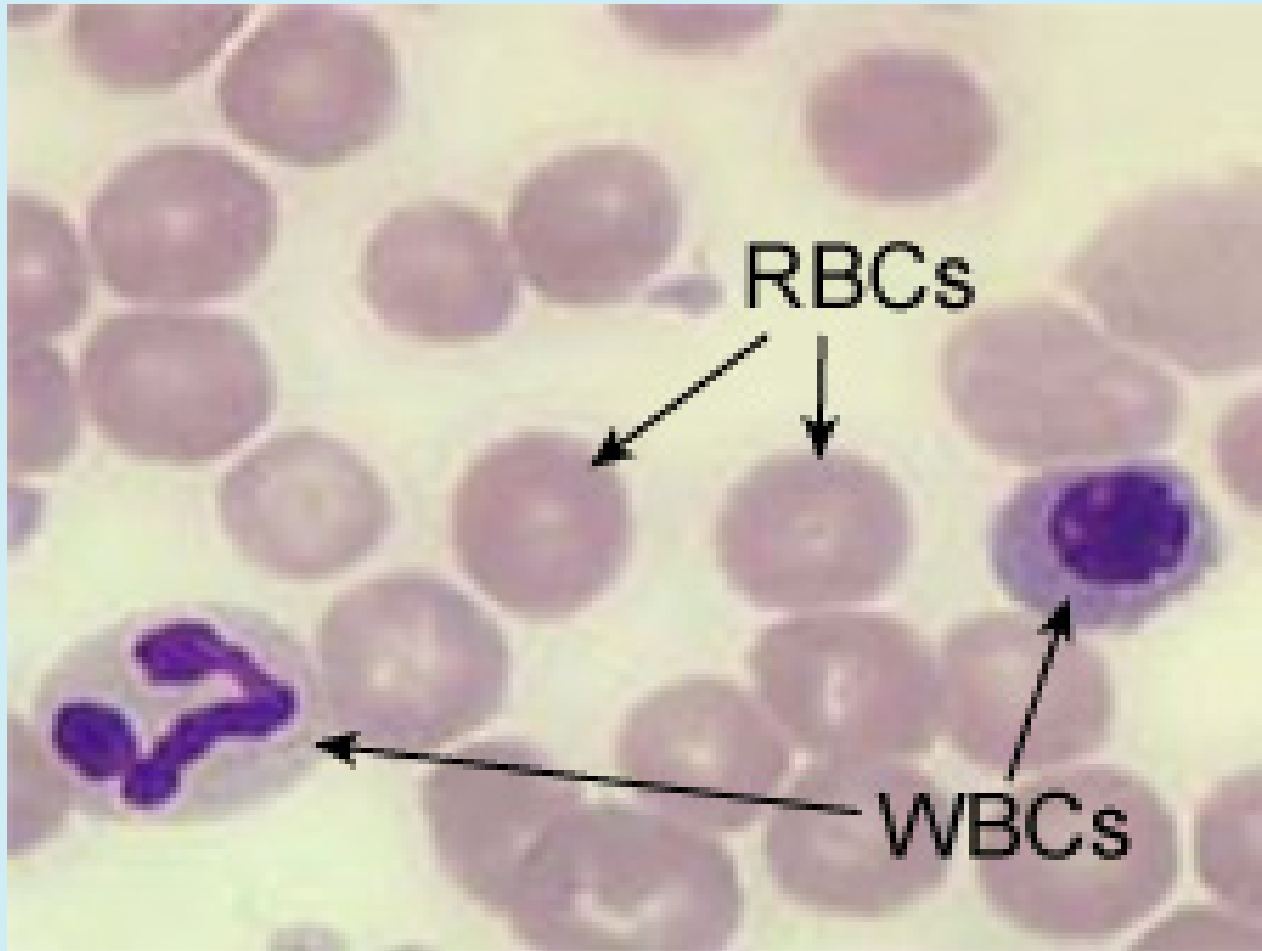
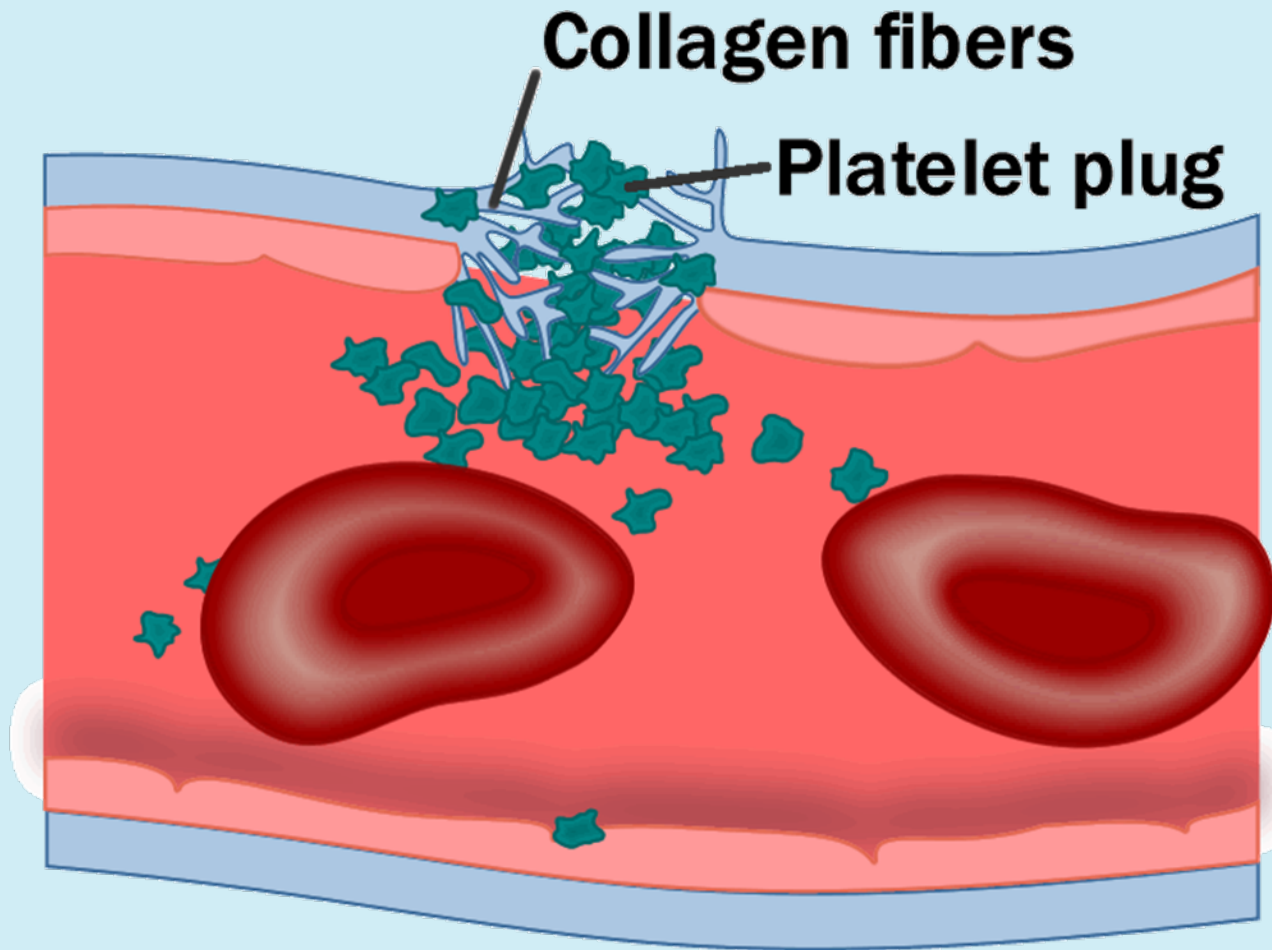


Image courtesy of the U.S. National Institutes of Health

Platelets

- **Irregularly-shaped, colorless bodies produced in the bone marrow**
- **Their sticky surface lets them, along with other substances, form clots to stop bleeding**
- **Only active when damage occurs to the circulatory system walls**

Hemostasis



Blood Volumes in the Body

- **Four to six liters of blood in the body**
 - **About 58% - Veins**
 - **About 13% - Arteries**
 - **About 12% - Pulmonary Vessels (Lungs)**
 - **About 9% - Heart**
 - **About 8% - Arterioles/Capillaries**

Blood in the Body

- **Understanding the breakdown may help explain the amount of blood at the crime scene**
- **ACTIVE FLOW – the heart is still pumping**
 - Injury to jugular – arterial spurting
- **PASSIVE FLOW – no blood pressure**
 - Blood flow following death
 - Gunshot to heart – blood pool near body

Forensic Significance Of Blood

- **Hemoglobin (RBC)**
 - Peroxidase-like activity can cleave H_2O_2
- **Blood Group Antigen (RBC)**
 - Bound to RBC membrane (ABO groups)
- **DNA (WBC)**
 - Found in cells with nucleus
- **Proteins (PLASMA)**
 - Serum used in species testing

Forensic Testing

- **Presumptive tests**
 - Indicates a substance is present
 - Not specific
- **Confirmatory tests**
 - Confirm a substance is present
 - Specific

Presumptive Tests

- **Kastle-Meyer (Phenolphthalein)**
- **Leucomalachite Green (LMG)**
- **Hemastix[®]**
- **Luminol**
- **Other tests:**
 - **Tetramethylbenzidine (TMB)**
 - **Benzidine**
 - **Ortho-tolidine**
 - **Ortho-toluidine**

Presumptive Tests

- **Catalytic Tests**

- **Based on the fact that hemoglobin (and some of its derivatives) exhibit a peroxidase activity**
 - **Tests based on this property are generally named after the compound undergoing oxidation (benzidine, TMB, etc.) or the discoverer (Kastle-Meyer, etc.)**
- **Oxidant (hydrogen peroxide) oxidizes a colorless reagent to a colored reagent**
- **Heme catalyzes this oxidation by cleaving an oxygen from hydrogen peroxide (H₂O₂)**

Kastle-Meyer Test – How to Make

- **Phenolphthalein + Potassium Hydroxide + Zinc**
 - **Reflux – boil / condense**
 - **Reduces phenolphthalein to phenolphthalin**
 - **Oxygen removed and combined with OH from potassium hydroxide – boils off as water**
 - **Becomes a colorless solution**
- **Working solution**
 - **Phenolphthalin + ethanol + zinc pellets**

Kastle-Meyer Test – How to Store

- **Amber bottle**
 - Light affects stability
- **Zinc pellets**
 - Binds free oxygen, prevents oxidation
- **Room temp (working solution)**
- **Refrigerate (stock solution)**

Kastle-Meyer Test – How to Perform

- **Place a small cutting, swabbing, or extract of the suspected bloodstain on filter paper**

- OR -

- **Swab the stain using a slightly moistened swab**

Kastle-Meyer Test – Three Step Test

- 1. Add two to three drops of ethanol to the stain or swabbing.**

Note: This will increase sensitivity by cleaning the area around the hemoglobin, better exposing the heme.

Kastle-Meyer Test – Three Step Test

- 2. Add two drops of reagent and wait for about five seconds.**

Note: This step aids in ruling out false positives due to the presence of chemical oxidants such as rust.

Kastle-Meyer Test – Three Step Test

- 3. Add two to three drops of 3% H₂O₂.**
 - If immediate color change to **PINK** – the test is **POSITIVE** for the possible presence of blood**
 - If no color change – blood is not present or is in too limited quantity for the test to detect.**

Note: The swab will eventually turn pink (even if negative) over time due to nature of oxidation reactions.

Kastle-Meyer Test – Limitations

- **Sensitivity**
 - 1 in 1000 on dried stains
- **Specificity**
 - Can weed out false positives between steps 2 and 3
 - Chemical oxidants, vegetable peroxidases
 - Will not detect differences in animal or human blood
- **Stability**
 - Relatively stable if the reagents are stored separately and refrigerated

Kastle-Meyer Test

Kastle-Meyer Video

Leucomalachite Green – How to Make

- **Malachite green + acetic acid + water + Zinc**
 - **Reflux – boil / condense**
 - **Becomes a colorless solution**
- **Working solution**
 - **LMG reagent + zinc pellets**

Leucomalachite Green – How to Store

- **Amber bottle**
- **Zinc pellets**
- **Relatively stable at room temp**

Leucomalachite Green – How to Perform

- **Place a small cutting, swabbing, or extract of the suspected bloodstain on filter paper**

- OR -

- **Swab the stain using a slightly moistened swab**

Leucomalachite Green – How to Perform

- 1. Add one to two drops of LMG reagent.**
 - A. Note color change.**
 - B. If there is a color change, the test is considered inconclusive.**

Leucomalachite Green – How to Perform

2. Add one to two drops of H₂O₂.
 - A. Note the results.
 - B. If color change to deep **green-blue**, the test is positive for the possible presence of blood.
 - C. If no color change the test is negative.

Leucomalachite Green – Limitations

- **Sensitivity**
 - About 1:1000
- **Specificity**
 - Chemical oxidants, vegetable peroxidases
 - Will not detect differences in animal or human blood
- **Stability**
 - Similar to KM

Leucomalachite Green

Leucomalacite Green Video

Hemastix[®]

- Reagent strips
- Bottle of 50 reagent strips
 - Store at room temp
- Test is based on the peroxidase activity of hemoglobin



Hemastix[®]

- Reagent on Hemastix[®] is diisopropylbenzene dihydroperoxide and 3,3',5,5'-tetramethylbenzidine (TMB)
- Color change ranges from **orange** to **green**
 - Possibly **blue** with higher concentrations of blood

Hemastix[®] – How to Perform

- 1. Slightly moisten the pad on the tip of the strip with water.**
- 2. Rub the damp pad on the stain in question.**
- 3. Note any color change within 60 seconds and compare to the chart on the bottle.**
 - More green indicates more hemoglobin**

Hemastix[®] – Limitations

- **Sensitivity = 0.015 - 0.062 mg/dL free hemoglobin**
- **Specificity**
 - **Chemical oxidants, vegetable peroxidases**
 - **Will not detect differences in animal or human blood**
- **Stability**
 - **Stable for about one year**
 - **Date stamped expiration date on bottle**

Hemastix[®]

Hemastix[®] Video

Luminol – How it Works

- **The iron in hemoglobin acts as a catalyst to cause a reaction between the luminol and H_2O_2**
- **Luminol loses nitrogen and hydrogen and gains oxygen**
- **This results in 3-aminophthalate which is energized and emits light**

Luminol – How to Make

- **Reagents needed:**
 - **Luminol (3-aminophthalhydrazide)**
 - **Sodium Perborate**
 - **Distilled water**
 - **Sodium Carbonate**

Luminol – How to Store

- **Spray bottle works best for testing**
- **Make each time you use it (daily)**

Luminol – How to Perform

- 1. Spray the luminol directly onto the stain in question.**
 - If the stain emits a light then the test result is POSITIVE for the possible presence of blood**
 - If there is no reaction the result is NEGATIVE**

Note: This test needs to be done in the dark to see the luminescence reaction, which can last for approximately 15 seconds.

Luminol – Limitations

- **Sensitivity**
 - **10-6 to 10-8 – most sensitive presumptive test**
- **Specificity**
 - **Many false positives – bleach, metals, chemical oxidants, vegetable peroxidases**
 - **Will not detect differences in animal or human blood**

Luminol – Limitations

- **Stability**
 - **Very unstable**
 - **About eight hour limit**
- **Mostly used at crime scene**
 - **Can dilute out stain (possibly too much for DNA analysis)**
 - **Used more for blood spatter, crime scene reconstruction**

Tetramethylbenzidine (TMB) – How to Make

- **3,3', 5,5 '-tetramethylbenzidine (TMB) +
glacial acetic acid**
 - **Easy to make compared to KM and LMG reagents**

Tetramethylbenzidine (TMB) – How to Store

- **Amber bottle**
 - Light affects stability
- **Refrigerate between uses**
 - Only good for about one week

Tetramethylbenzidine (TMB) – How to Perform

- **Place a small cutting, swabbing, or extract of the suspected bloodstain on filter paper**

- OR -

- **Swab the stain using a slightly moistened swab**

Tetramethylbenzidine (TMB) – How to Perform

- 1. Add one drop of TMB solution.**
- 2. Add one drop of 3% H₂O₂.**
- 3. Detect color change:**
 - If the stain turns blue-ish green, the test result is POSITIVE for the possible presence of blood**
 - NEGATIVE if no color change**

Tetramethylbenzidine (TMB) – Limitations

- **Sensitivity**
 - **1:10,000 on dried stains**
- **Specificity**
 - **Not as specific as KM test**
 - **False positives to vegetable peroxidases, bleach, potassium permanganate**
 - **Will not detect differences in animal or human blood**

Tetramethylbenzidine (TMB) – Limitations

- **Stability**
 - Very unstable – one week maximum
 - Loses sensitivity by a factor of 10 after one day
- **Safety**
 - Mutagen

Other Tests

- **Benzidine**
- **Ortho tolidine (o-tolidine) –
3,3'-Dimethylbenzidine**
 - **Increased sensitivity, decreased specificity, and same stability when compared to Kastle-Meyer**
 - **Rarely used due to safety concerns – carcinogenic**

ABAcad[®] HemaTrace[®]

- **Tests for human hemoglobin (Hb)**
- **If human Hb is present – reacts with a mobile monoclonal anti-human HB antibody**
- **Forms a mobile Ag-Ab complex**
- **This migrates to the “T” zone**

ABAcad[®] HemaTrace[®]

- **In the “T” zone – polyclonal antihuman HB antibodies**
- **Forms Ab-Ag-Ab complex**
- **Antibodies tagged with pink dye – upon aggregation at “T” zone – pink line**
- **Control zone – has immobile anti-immunoglobulin which binds excess antihuman HB antibodies – form a pink line**

ABAcad[®] HemaTrace[®] – How to Perform

- **Extract cutting from stain in about 300 µl buffer**
- **Leave one to five minutes**
- **Add 150 µl of sample to the “S” well of the test card**
- **Wait 10 minutes, then read results**
- **Pink line in “T” and “C” zones = POSITIVE**
- **Pink line in only “C” zone = NEGATIVE**

ABAcad[®] HemaTrace[®] – Limitations

- **High Dose Hook Effect – can give a false negative**
 - Occurs with excess hemoglobin, which binds to the stationary antihuman HB Antibody in the “T” area
 - This prevents the mobile Ab-Ag complex from binding
 - Results in no pink line = **NEGATIVE**
 - **FALSE NEGATIVE**
- **Not a confirmatory test – gives positive result with ferret blood**

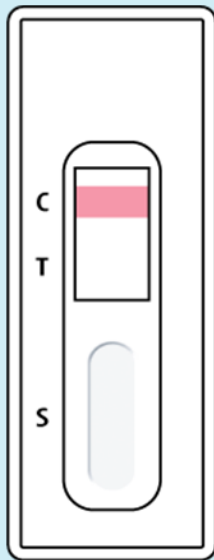
ABAcad[®] HemaTrace[®]



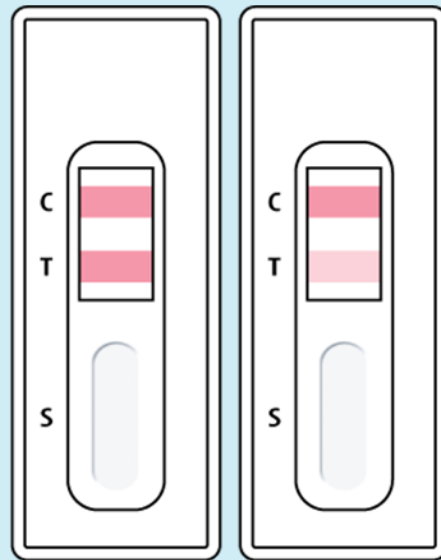
HemaTrace Video

Seratec[®] HemDirect Hemoglobin Assay

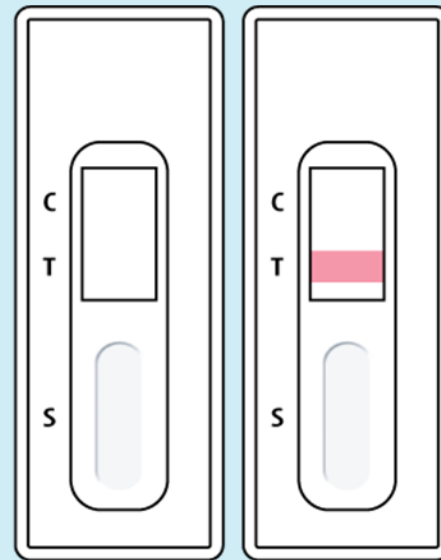
- Tests for human hemoglobin (Hb)
 - Similar to the ABACard[®] HemaTrace[®] test



Negative



Positive



Invalid

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Controls

- **Positive**
- **Negative**
- **Substrate**

Positive Controls

- **Used to determine if the tests are working properly**
 - **A quality control check**
- **This is documented in the case file**
- **Example: Known blood on cloth or a swab**

Negative Controls

- **Used to determine if the reagents are contaminant free and working properly**
 - **A quality control check**
- **This is documented in the case file**
- **Example: Unstained cloth or a swab**

Substrate Controls

- **Used to determine if the substrate is interfering with the test**
 - Troubleshooting
- **This is documented in the case file**
- **Example: Unstained area adjacent to the questioned stain being tested**

Questions?