

SCREENING TESTS FOR BLOOD**8.1 PURPOSE**

8.1.1: To perform screening tests for the presence of blood in Forensic samples.

A. Theory

1. The Kastle–Meyer (KM) test is a chemical screening test for the presence of blood in which the chemical indicator phenolphthalein is used to detect the possible presence of hemoglobin. In the presence of an oxidant (H_2O_2), phenolphthalein relies on the peroxidase-like activity of the heme component of hemoglobin in blood to catalyze phenolphthalin (colorless reduced form) into phenolphthalein (oxidized form), which is visible as a bright pink color.
2. The o-Tolidine chemical screening test for the presence of blood also relies on the peroxidase-like activity of the heme component of hemoglobin in blood. The heme catalyzes the reaction that causes the solution's transparent beige color (reduced form) to change to a blue/blue-green color (oxidized form) in the presence of an oxidant (H_2O_2).

B. Limitations

1. Chemical oxidants will cause a color change before the addition of the hydrogen peroxide. Therefore, it is important to employ the two-step method.
2. False positive results may occur if the result is not read within 10 seconds.
3. False positive results may occur in the presence of plant peroxidases.
4. These tests can neither determine nor detect the difference between human blood and animal blood.

8.1.2: To prepare reagents for blood screening tests and to perform quality control on prepared reagents.

8.2 RESPONSIBILITY

8.2.1: Test Procedure – Personnel qualified to perform Forensic Biology duties.

8.2.2: Preparation/QC Procedure – Personnel qualified to perform Forensic Biology duties.

- A. Ordering information is maintained electronically and/or in a logbook in the Forensic Biology Unit.
- B. New chemicals and reagents are purchased according to GL-6 (Purchasing).
- C. Refer to the following for additional information:
 1. Chemical and Reagent Inventories located in Appendix 3
 2. FB SOP-21 (General and Reagent QC)

8.3 SAFETY

Use appropriate measures for the proper handling of biohazardous material, o-Tolidine solution, ethanol, glacial acetic acid and potassium hydroxide according to GL-2 (Safety Manual) and the Safety Data Sheets.

8.4 DEFINITIONS/ABBREVIATIONS

- A. KM: Kastle-Meyer
- B. o-Tol: o-Tolidine
- C. QRW(s): Quality Record Worksheet(s) (Appendix 1)

8.5 TEST PROCEDURE

These tests will be performed at the discretion of the examiner, with input from the Unit Lead(s) when applicable, based on the submitting agency requests, case information and the condition of the evidence.

- A. The KM test is conducted routinely. The o-Tolidine test may be used if the sample is limited/compromised or if a negative/inconclusive/failed KM test result is obtained.
- B. A sample is considered limited when it appears to be of low quantity and compromised when it appears to be in poor condition. The conditions the evidence may have been exposed to prior to submission shall be considered when assessing the sample tested and/or collected.
- C. For previously tested items (examples: field tested, cold case samples, Latent Print blood enhancement reagent):
 - 1. Swabs: Blood screening tests may be omitted.
 - 2. Other evidence:
 - a. If the item of evidence has visible reddish-brown stains, then blood screening tests may be performed.
 - b. If an area on an item of evidence is designated as positive from previous testing but no reddish-brown staining is visible, then collect a swabbing of the indicated area. Do not perform blood screening tests on the area or swabbing. Forward the swabbing for DNA analysis.
 - 3. See FB SOP-01 (Evidence Examination and Sample Collection Guidelines) for evidence previously examined by the Latent Print Unit.

8.5.1: Materials

- A. Phenolphthalin solution (for the KM test)
- B. o-Tolidine solution
- C. 3.0% Hydrogen peroxide
- D. Controls: positive (known bloodstain) and negative (blank substrate)
- E. dH₂O
- F. Cotton swabs or spot plates

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- G. Forceps
- H. Scissors/scalpel(s)

8.5.2: Procedure

- A. Record the reagent(s) used on the General Reagent Sheet (FBQR-09) and appropriate QRW(s).
- B. Test a positive control and negative control according to the following procedure (steps 8.5.2.C - 8.5.2.F) prior to testing the questioned sample. If using dH₂O for the testing of questioned samples, the dH₂O must be tested with the positive and negative controls.
 - 1. If the controls yield the appropriate results, record on the appropriate QRW(s) and test the questioned samples.
 - 2. If the controls do not yield the appropriate results, review the procedure and retest the controls prior to beginning analysis on casework samples. If the controls still do not yield the appropriate results, then inform the Unit Lead to try to determine the root cause.
 - 3. If the phenolphthalin solution of the KM reagent has turned pink, do not use. Discard and inform a Forensic Biology Lead.
- C. Samples may be prepared for testing as follows:
 - 1. Indirect testing will typically be conducted. Moisten a cotton swab with dH₂O and lightly swab the questioned stain or area.
 - 2. Direct testing may be conducted (without dH₂O) as follows:
 - a. Cut a portion of the stained fabric, swab or other substrate and place the cutting into a spot plate or other device.
 - b. The stain may be scraped from a surface (typically non-absorbent) for direct testing.

Note: If a sample is considered limited (see 8.5.B), it may be forwarded for DNA analysis without conducting a blood screening test.
- D. Add one drop of phenolphthalin or o-Tolidine solution to the portion of questioned sample. Caution: Testing with o-Tolidine solution should be performed under the hood.
- E. If no color change occurs, add one (1) drop of 3% H₂O₂.
- F. Observe any color change within 10 seconds.
- G. Record the results on the appropriate QRW(s).

- H. If the quantity of stain is insufficient to perform a confirmatory test, or the case scenario does not warrant further serological testing, a positive chemical screening test is sufficient to forward the stain directly for DNA analysis.

8.5.3: Results and Conclusions

A. Positive

1. If blood or "other peroxidase-type material" is present, a color change will occur after the addition of 3% H₂O₂. Phenolphthalin (KM test) will turn pink; o-Tolidine will turn blue/blue-green.
2. Positive results may be designated and recorded as follows:
 ↓/vw(+) = very weak positive = very light pink or blue/blue-green
 w(+) = weak positive = light pink or blue/blue-green
 (+) = positive = pink or blue/blue-green
 ↑/s(+) = strong positive = dark pink or blue/blue-green

The description of a positive result may vary between examiners. This variation is acceptable since all descriptions designate a positive result. Recording the description of a positive result may be helpful in interpreting the result(s) obtained from additional serological testing and/or DNA analysis.

3. Suggested Report Wording:

a. When no blood confirmatory test conducted:

Testing Performed	Results	Conclusion
Screening – Blood	Positive	Blood indicated ¹
Comment: ¹ No further serological testing was conducted at this time.		

b. When confirmatory test conducted:

Testing Performed	Results	Conclusion
Screening – Blood	Positive	Blood indicated

c. [] gave a positive result(s) with a color screening test(s) for the presence of blood.

B. Negative

1. A negative reaction will show no color change within 10 seconds of the addition of 3% H₂O₂, indicating no blood was detected.
2. Suggested Report Wording:

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a.

Testing Performed	Results	Conclusion
Screening – Blood	Negative	Blood not detected

- b. *A color screening test(s) for the presence of blood was/were performed on [].
Blood was not detected with this/these test(s).*

C. Inconclusive

1. If a pink or blue/blue-green color change could not be determined after the addition of 3% H₂O₂ (i.e. when there is interference from the substrate).

2. Suggested Report Wording:

a.

Testing Performed	Results	Conclusion
Screening – Blood	Indeterminate	Inconclusive ¹

Comment:

¹Due to an indeterminate result and/or substrate interference, this test was determined to be inconclusive.

- b. *A color screening test for the presence of blood was performed on [].
Due to an indeterminate result and/or substrate interference, this test was determined to be inconclusive.*

3. Record the reason a result is determined to be inconclusive on the appropriate QRW(s).

D. Failed

1. The appearance of a pink or blue/blue-green color change prior to the addition of 3% H₂O₂ indicates the presence of a chemical oxidant in the stain.

2. Suggested Report Wording:

a.

Testing Performed	Results	Conclusion
Screening – Blood	Failed Test	No conclusion possible

- b. *A color screening test(s) for the presence of blood was/were performed on [].
Due to the failure of this/these test(s), no conclusion(s) is/are possible.*

2. Record the reason the test failed on the appropriate QRW(s).

- E. It should be noted that any result above does not preclude the sample from being forwarded for further serological testing or DNA analysis, as determined on a case-by-case basis.

- F. See FB SOP-05 (Case Records and Reports) for additional information.

8.6 PREPARATION/QC PROCEDURE

8.6.1: Phenolphthalin Stock Solution

Materials

- A. Phenolphthalin 2g
- B. Potassium hydroxide 20g
- C. dH₂O 100ml
- D. Granular Zinc 20g

If stock remains, the control date will be one (1) year from the date of preparation.

Procedure

- A. Dissolve potassium hydroxide in dH₂O. Note: The solution will be warm from the reaction.
- B. Add granular zinc followed by phenolphthalin. Solution turns pink immediately.
- C. Swirl solution until it becomes colorless. The granular zinc stabilizes the solution, preventing it from becoming oxidized and turning pink.
- D. If stock remains, place in a brown bottle containing zinc.
- E. Discard on the control date, or sooner, if the colorless solution turns pink.

8.6.2: Phenolphthalin Working Solution

Materials

- A. Phenolphthalin stock solution 1 part
- B. Ethanol 4 parts
- C. Granular Zinc ~15g

The control date will be six (6) months from the date of preparation.

Procedure

- A. Dilute stock solution 1:5 with ethanol (1 part plus 4 parts).
- B. Place working solution in brown dropper bottles containing zinc. The granular zinc stabilizes the solution, preventing it from becoming oxidized and turning pink.
- C. Test each new batch of the working solution before use according to the test procedure and the KM Reagent Log Sheet. Record the required information.

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D. If the appropriate results are not obtained, discard the reagent, review the procedure, make new reagent and retest. If the reagent still does not yield the appropriate results, then inform the Unit Lead to try to determine the root cause.

E. If the reagent is acceptable for use, record the solution, lot # (date of preparation), control date and examiner's initials on the stock and dropper bottles and store in the refrigerator.

The KM reagent is acceptable for use when a positive result is obtained with the blood control and a negative result is obtained with the blank/negative control.

F. Discard the working solution on the control date (not to exceed the control date of the stock solution), or sooner, if it turns pink.

G. Discard/replace the phenolphthalin according to manufacturer's expiration date or according to 21.4.3.E in FB SOP-21 (General Chemical and Reagent QC).

Manufacturer's expiration dates with only month and year indicated (i.e. 04/2014) expire the last day of the month noted.

8.6.3: o-Tolidine Solution

Materials

- | | | |
|----|---------------------|--------|
| A. | o-Tolidine | 1.6g |
| B. | Ethanol | 40.0ml |
| C. | Glacial acetic acid | 30.0ml |
| D. | dH ₂ O | 30.0ml |

The control date will be one (1) year from the date of preparation.

Procedure

- A. Mix all materials together and place into a brown dropper bottle.
- B. Test each new batch of reagent before use according to the test procedure and the o-Tolidine Reagent Log Sheet. Record the required information.
- C. If the appropriate results are not obtained, discard the reagent, review the procedure, make new reagent and retest. If the reagent still does not yield the appropriate results, then inform the Unit Lead to try to determine the root cause.
- D. If the reagent is acceptable for use, record the solution, lot # (date of preparation), control date and examiner's initials on the dropper bottle and store in the refrigerator.

The o-Tol reagent is acceptable for use when a positive result is obtained with the blood control and a negative result is obtained with the blank/negative control.

- E. Discard the o-Tolidine solution on the control date, or sooner, if it changes from transparent beige to a dark brown color.
- F. Discard/replace the o-Tolidine according to manufacturer's expiration date or according to 21.4.3.E in FB SOP-21 (General Chemical and Reagent QC).

Manufacturer's expiration dates with only month and year indicated (i.e. 04/2014) expire the last day of the month noted.

8.6.4: 3 % Hydrogen Peroxide

- A. This chemical is purchased from an approved outside vendor.
- B. Test the new manufacturer's lot before use in casework according to the test procedure and the KM and o-Tolidine Reagent Log Sheets. Record the required information.
- C. If the appropriate results are not obtained, review the procedure and repeat the test. If the reagent still does not yield the appropriate results, then inform the Unit Lead to try to determine the root cause.
- D. If the lot is acceptable for use, record the date received, date opened and examiner's initials on the stock bottles and store in the refrigerator.

The 3% Hydrogen Peroxide is acceptable for use with KM and o-Tol reagents when a positive result is obtained with the blood control and a negative result is obtained with the blank/negative control.

- E. Aliquot into dropper bottles labeled with the chemical, lot #, manufacturer's expiration date, fill date and initials on the dropper bottles.
- F. Discard according to the manufacturer's expiration date or according to 21.4.3.E in FB SOP-21 (General Chemical and Reagent QC) or sooner if a decrease in reaction activity is observed while testing the controls.

Manufacturer's expiration dates with only month and year indicated (i.e. 04/2014) expire the last day of the month noted.

8.7 REFERENCES

- A. Cox, M., "A study of the Sensitivity and Specificity of Four Presumptive Tests for Blood", *Journal of Forensic Sciences*, JFSCA, Vol. 36, No. 5, Sept. 1991, pp. 1503-1511.
- B. Li, Richard, Forensic Biology, Second Edition, CRC Press, FL, 2015:
Chapter 2: "Crime Scene Bloodstain Pattern Analysis", pp. 44-51 (Types of Bloodstain Patterns)
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Chapter 12: "Identification of Blood", pp. 231-239
- C. Tobe, S.S., N. Watson, N. N. Daeid, "Evaluation of Six Presumptive Tests for Blood, Their Specificity, Sensitivity, and Effect on High Molecular-Weight DNA, *J. of Forensic Sci.*, January 2007, Vol. 52, No. 1, pp. 102-109.
- D. GL-2 (Safety Manual)
- E. GL-6 (Purchasing)
- F. Safety Data Sheets