

# HORSE HEMOGLOBIN

## Immunoperoxidase Assay for Determination of Hemoglobin in Horse Samples

### DIRECTIONS FOR USE

Version 1.0 -- 1

For Research Use Only, NOT for Diagnostic Purposes

**Please Read this Package Insert Completely Before Using This Product**

### INTENDED USE

The Hemoglobin test kits are a highly sensitive two-site enzyme linked immunoassay (ELISA) for measuring Hemoglobin in biological samples of horses.

### INTRODUCTION

Hemoglobin (HM) is the iron-containing oxygen transport protein in the red blood cells. It picks up oxygen in the lungs and delivers it to the tissues to maintain the viability of cells. Each subunit of hemoglobin is a globular protein with an embedded heme group. Each heme group contains an iron atom and is responsible for the binding of oxygen. Decrease of hemoglobin leads to symptoms of anemia.

### PRINCIPLE OF THE ASSAY

The principle of the double antibody sandwich ELISA is represented in Figure 1. In this assay the Hemoglobin present in samples reacts with the anti-Hemoglobin antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal of unbound proteins by washing, anti-HM antibodies conjugated with horseradish peroxidase (HRP), are added. These enzyme-labeled antibodies form complexes with the previously bound HM. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of HM in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of HM in the test sample. The quantity of HM in the test sample can be interpolated from the standard curve constructed from the standards, and corrected for sample dilution.

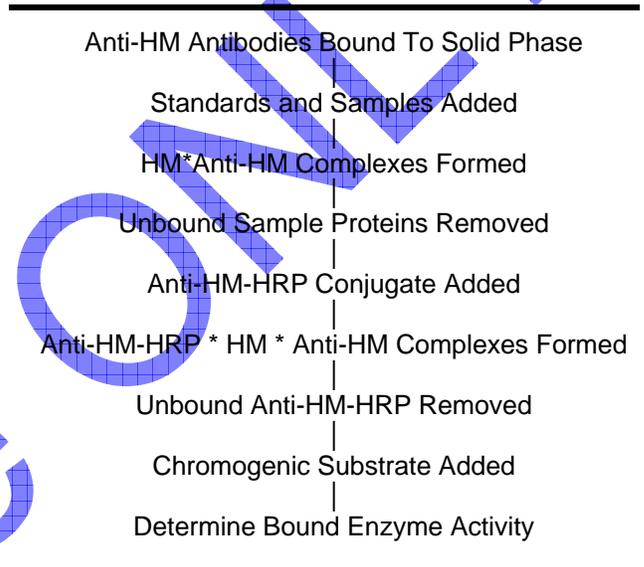


Figure 1.

### REAGENTS (Quantities sufficient for 96 determinations)

- 1. DILUENT CONCENTRATE (Running Buffer)**  
One bottle containing 50 ml of a 5X concentrated diluent running buffer.
- 2. WASH SOLUTION CONCENTRATE**  
One bottle containing 50 ml of a 20X concentrated wash solution.
- 3. ENZYME-ANTIBODY CONJUGATE 100X**  
One vial containing 150  $\mu$ L of affinity purified anti-Horse Hemoglobin antibody conjugated with horseradish peroxidase in a stabilizing buffer.
- 4. CHROMOGEN-SUBSTRATE SOLUTION**  
One vial containing 12 mL of 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide in citric acid buffer at pH 3.3.

5. STOP SOLUTION  
One vial containing 12 ml 0.3 M sulfuric acid.

**WARNING: Avoid contact with skin.**

6. ANTI-HORSE HEMOGLOBIN ELISA MICRO PLATE  
Twelve removable eight (8) well micro well strips in well holder frame. Each well is coated with affinity purified anti-Horse HM.

7. HORSE HEMOGLOBIN CALIBRATOR  
One vial containing Horse Hemoglobin calibrator.

**FOR IN VITRO USE ONLY**

**REAGENT PREPARATION**

1. DILUENT CONCENTRATE  
The Diluent Solution supplied is a 5X Concentrate and must be diluted 1/5 with distilled or deionized water (1 part buffer concentrate, 4 parts dH2O).

2. WASH SOLUTION CONCENTRATE  
The Wash Solution supplied is a 20X Concentrate and must be diluted 1/20 with distilled or deionized water (1 part buffer concentrate, 19 parts dH2O). Crystal formation in the concentrate is not uncommon when storage temperatures are low. Warming of the concentrate to 30-35°C before dilution can dissolve crystals.

3. ENZYME-ANTIBODY CONJUGATE  
Calculate the required amount of working conjugate solution for each microtitre plate test strip by adding 10 µL Enzyme-Antibody Conjugate to 990 µL of 1X Diluent for each test strip to be used for testing. Mix uniformly, but gently. Avoid foaming.

4. CHROMOGEN-SUBSTRATE SOLUTION  
Ready to use as supplied.

5. STOP SOLUTION  
Ready to use as supplied.

6. ANTI-HORSE HEMOGLOBIN ELISA MICRO PLATE  
Ready to use as supplied. Unseal Microtiter Pouch and remove plate from pouch. Remove all strips and wells that will not be used in the assay and place back in pouch and re-seal.

7. HORSE HEMOGLOBIN CALIBRATOR  
The calibrator is now at a concentration of 344 ug/ml  
**Horse Hemoglobin standards need to be**

**prepared immediately prior to use (see chart below).** Mix well between each step. Avoid foaming.

Standard	ng/ml	Volume added to 1x Diluent	Volume of 1x Diluent
A	3440	5 µl Horse HM Calibrator	495 µl
6	400	100 µl standard 6	760 µl
5	200	300 µl standard 5	300 µl
4	100	300 µl standard 4	300 µl
3	50	300 µl standard 3	300 µl
2	25	300 µl standard 2	300 µl
1	12.5	300 µl standard 1	300 µl
0	0		500 µl

**STORAGE AND STABILITY**

The expiration date for the package is stated on the box label.

1. DILUENT  
The 5X Diluent Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions should be stored at 4-8°C.

2. WASH SOLUTION  
The 20X Wash Solution Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions can be stored at room temperature (16-25°C) or at 4-8°C.

3. ENZYME-ANTIBODY CONJUGATE  
Undiluted horseradish peroxidase anti-HM conjugate should be stored at 4-8°C and **diluted immediately prior to use.** The working conjugate solution is stable for up to 8 hours.

4. CHROMOGEN-SUBSTRATE SOLUTION  
The Substrate Solution should be stored at 4-8°C and is stable until the expiration date.

5. STOP SOLUTION  
The Stop Solution should be stored at 4-8°C and is stable until the expiration date.

6. ANTI-HORSE HEMOGLOBIN ELISA MICRO PLATE  
Anti-Horse HM coated wells are stable until the expiration date, and should be stored at 4-8°C in sealed foil pouch with desiccant pack.

## 7. HORSE HEMOGLOBIN CALIBRATOR

Long Term Storage: Upon receipt, aliquot the calibrator and store them frozen. They will be stable until expiration date. Short Term Storage: the calibrator is stable for up to 14 days at 4°C. The working standard solutions should be prepared immediately prior to use and are stable for up to 8 hours.

### INDICATIONS OF INSTABILITY

If the test is performing correctly, the results observed with the standard solutions should be within 20 % of the expected values.

### SPECIMEN COLLECTION AND HANDLING

Blood should be collected by venipuncture. The serum should be separated from the cells after clot formation by centrifugation. For plasma samples, blood should be collected into a container with an anticoagulant and then centrifuged. Care should be taken to minimize hemolysis, excessive hemolysis can impact your results. Assay immediately or aliquot and store samples at -20°C. Avoid repeated freeze-thaw cycles.

#### 1. Precautions

For any sample that might contain pathogens, care must be taken to prevent contact with open wounds.

#### 2. Additives and Preservatives

No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.

#### 3. Known interfering substances

Azide and thimerosal at concentrations higher than 0.1% inhibits the enzyme reaction.

### MATERIAL PROVIDED

See "REAGENTS"

### MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipette (2 µL to 200 µL) for making and dispensing dilutions
- Test tubes
- Microtitre washer/aspirator
- Distilled or Deionized H<sub>2</sub>O
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- Microtitre Plate reader
- Assorted glassware for the preparation of reagents and buffer solutions
- Timer
- Vortex mixer

### ASSAY PROTOCOL

#### DILUTION OF SAMPLES

The assay for quantification of Hemoglobin in samples requires that each test sample be diluted before use. For a single step determination a dilution of 1/5,000 is appropriate for most serum/plasma samples. For absolute quantification, samples that yield results outside the range of the standard curve, a lesser or greater dilution might be required.

1. To prepare a 1/5,000 dilution of sample, transfer 5 µL of sample to 495 µL of 1X diluent. This gives you a 1/100 dilution. Next, add 10 µL of your 1/100 diluted sample to 490 µL of 1X diluent. You now have a 1/5,000 dilution of your sample. Mix thoroughly at each stage.

#### PROCEDURE

1. **Bring all reagents to room temperature before use.**

2. Pipette 100 µL of
  - Standard 0 (0.0 ng/ml) in duplicate
  - Standard 1 (12.5 ng/ml) in duplicate
  - Standard 2 (25 ng/ml) in duplicate
  - Standard 3 (50 ng/ml) in duplicate
  - Standard 4 (100 ng/ml) in duplicate
  - Standard 5 (200ng/ml) in duplicate
  - Standard 6 (400ng/ml) in duplicate

3. Pipette 100 µL of sample (in duplicate) into pre designated wells.

4. Incubate the micro titer plate at 22°C (room temperature) for thirty (30 ± 2) minutes. Keep plate covered and level during incubation.

5. Following incubation, aspirate the contents of the wells.

6. Completely fill each well with appropriately diluted Wash Solution and aspirate. Repeat three times, for a total of four washes. If washing manually: completely fill wells with wash buffer, invert the plate then pour/shake out the contents in a waste container. Follow this by sharply striking the wells on absorbent paper to remove residual buffer. Repeat 3 times for a total of four washes.

7. Pipette 100  $\mu$ L of appropriately diluted Enzyme-Antibody Conjugate to each well. Incubate at 22°C (room temperature) for twenty (20  $\pm$  2) minutes. Keep plate covered in the dark and level during incubation.

8. Wash and blot the wells as described in Steps 5/6.

9. Pipette 100  $\mu$ L of TMB Substrate Solution into each well.

10. Incubate in the dark at room temperature for precisely ten (10) minutes.

11. After ten minutes, add 100  $\mu$ L of Stop Solution to each well.

12. Determine the absorbance (450 nm) of the contents of each well. Calibrate the plate reader to air.

#### STABILITY OF THE FINAL REACTION MIXTURE

The absorbance of the final reaction mixture can be measured up to 2 hours after the addition of the Stop Solution. However, good laboratory practice dictates that the measurement be made as soon as possible.

#### RESULTS

1. Subtract the average background value from the test values for each sample.

2. Using the results observed for the standards construct a Standard Curve. The appropriate curve

fit is that of a four-parameter logistics curve. A second order polynomial (quadratic) or other curve fits may also be used.

3. Interpolate test sample values from standard curve. Correct for sera dilution factor to arrive at the Hemoglobin concentration in original samples.

#### LIMITATION OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the information contained in the package insert instructions and with adherence to good laboratory practice.

2. Factors that might affect the performance of the assay include proper instrument function, cleanliness of glassware, quality of distilled or deionized water, and accuracy of reagent and sample pipettings, washing technique, incubation time or temperature.

3. Do not mix or substitute reagents with those from other lots or sources.

Manufactured by:



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