

# **ab205071 – Dog Hemoglobin ELISA Kit**

Instructions for Use

For the quantitative measurement of Hemoglobin in biological fluids of dogs.

This product is for research use only and is not intended for diagnostic use.

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## 1. BACKGROUND

The Hemoglobin ELISA (ab205071) test kit is a highly sensitive two-site enzyme linked immunoassay (ELISA) for measuring Hemoglobin biological fluids of dogs.

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-Hemoglobin antibodies conjugated with horseradish peroxidase (HRP), are added. These enzyme-labeled antibodies form complexes with the previously bound Hemoglobin. 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 Hemoglobin in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of Hemoglobin in the test sample. The quantity of Hemoglobin in the test sample can be interpolated from the standard curve constructed from the standards, and corrected for sample dilution.

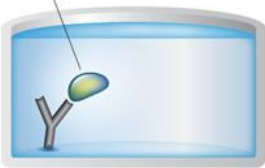
## 2. ASSAY SUMMARY

### Primary capture antibody



Remove appropriate number of antibody coated well strips. Equilibrate all reagents to room temperature. Prepare all the reagents, samples, and standards as instructed.

### Sample



Add standard or sample solution to each well. Incubate at room temperature.

### HRP conjugated antibody



Aspirate and wash each well. Add prepared HRP labeled secondary detector antibody. Incubate at room temperature.

### Substrate **Colored product**



Aspirate and wash each well. Add Chromogen Substrate Solution to each well. Measure immediately.

## 3. PRECAUTIONS

**Please read these instructions carefully prior to beginning the assay.**

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

## 4. STORAGE AND STABILITY

**Store kit at +4°C immediately upon receipt.**

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 9. Reagent Preparation.

## 5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage condition (After Preparation)
5X Diluent Concentrate	50 mL	4°C	4°C
20X Wash Buffer Concentrate	50 mL	4°C	4°C
100X Enzyme Antibody Conjugate	150 µL	4°C	4°C*
Chromogen Substrate Solution	12 mL	4°C	4°C
Stop Solution	12 mL	4°C	4°C
Anti-Dog Hemoglobin ELISA Microplate	1	4°C	4°C
Dog Hemoglobin Calibrator (Lyophilized)	1 Vial	4°C	4°C

\* **Only dilute immediately prior to use.** The working conjugate solution is stable for up to 1 hour when stored in the dark.

### **6. MATERIALS REQUIRED, NOT SUPPLIED**

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at 450 nm.
- Microplate washer/ aspirator
- Precision pipettes to deliver 2  $\mu$ L to 200  $\mu$ L volumes.
- Timer
- Distilled or deionized water.
- Log-log graph paper or computer and software for ELISA data analysis.
- Tubes to prepare standard or sample dilutions.
- Assorted glassware for the preparation of reagents and buffer solutions.

### **7. LIMITATIONS**

- 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.
- Factors that might affect the performance of the assay include proper instrument function, cleanliness of glassware, quality of distilled or deionized water, accuracy of reagent and sample pipetting, washing technique, incubation time and/or temperature.
- Do not mix or substitute reagents with those from other lots or sources.

### 8. TECHNICAL HINTS

- Samples which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Completely aspirate all solutions and buffers during wash steps. When preparing your standards, it is critical to briefly spin down the vial first. The powder may adhere to the cap and not be included in the standard solution resulting in an incorrect concentration. Be sure to dissolve the powder thoroughly when reconstituting. After adding Assay Diluent to the vial, we recommend inverting the tube a few times, then flick the tube a few times, and then spin it down; repeat this procedure 3-4 times. This is an effective technique for thorough mixing of the standard without using excessive mechanical force.
- Do not vortex the standard during reconstitution, as this will destabilize the protein.
- Once your standard has been reconstituted, it should be used right away or else frozen for later use.
- Keep the standard dilutions on ice during preparation, but the ELISA procedure should be done at room temperature.
- Be sure to discard the working standard dilutions after use – they do not store well.
- **This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Scientific Support staff with any questions.**

## 9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18-25°C) prior to use.

### 9.1 **5X 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 dH<sub>2</sub>O).

### 9.2 **20X Wash Buffer 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 dH<sub>2</sub>O). Crystal formation in the concentrate is not uncommon when storage temperatures are low. If crystals are observed, warm the buffer concentrate to 30-35°C to dissolve the crystals before dilution.

### 9.3 **100X Enzyme-Antibody Conjugate**

Calculate the required amount of 1X Enzyme-Antibody Conjugate solution for each microplate 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 and avoid foaming. The working conjugate solution is stable for up to 1 hour when stored in the dark.

### 9.4 **Chromogen Substrate Solution**

Ready to use as supplied.

### 9.5 **Stop Solution**

Ready to use as supplied.

### 9.6 **Anti-Dog Hemoglobin ELISA Microplate**

Ready to use as supplied. Unseal Microplate pouch and remove plate. Store stripwells that will not be used in this experiment by placing back in pouch and re-seal with the supplied desiccant.



### 9.7 **Dog Hemoglobin Calibrator**

Reconstitute the Dog Hemoglobin Calibrator with distilled or de-ionized water as specified on the vial and mix gently until dissolved. The amount of calibrator is shown on the vial, and after reconstitution will have a concentration of  $X \mu\text{g/mL}$ , where  $X$  is the amount on the vial (the reconstituted calibrator should be aliquoted and stored frozen if future use is intended).

## 10. STANDARD PREPARATIONS

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use.

**10.1** The liquid Dog Hemoglobin Calibrator should be used within 1 hour or aliquot and store at  $-20^{\circ}\text{C}$  for future use. Avoid multiple freeze-thaw cycles. **The calibrator is provided at the concentration stated on the vial.**

10.2 Label tubes numbers 1-7.

10.3 Prepare **Standard #1** by adding the appropriate volume of 1X Diluent Solution and Dog Hemoglobin Calibrator (derived below) to tube #1. Mix well between each step. Avoid foaming.

\*Example:

**NOTE: This example is for demonstration purposes only. Please remember to check your calibrator vial for the actual concentration of calibrator provided.**

$C_S$  = Starting concentration of reconstituted Dog Hemoglobin Calibrator (variable e.g.  $8.3\ \mu\text{g/mL}$ / $8,300\ \text{ng/mL}$ )

$C_F$  = Final concentration of Dog Hemoglobin Calibrator for the assay procedure ( $200\ \text{ng/mL}$ )

$V_A$  = Total volume of stock Dog Hemoglobin Calibrator to dilute (e.g.  $20\ \mu\text{L}$ )

$V_D$  = Total volume of 1X Diluent Solution required to dilute Dog Hemoglobin Calibrator to prepare **Standard #1**

$V_T$  = Total volume of **Standard #1**

## ASSAY PREPARATION

Calculate the dilution factor ( $D_F$ ) between stock calibrator and the **Standard #1** final concentration:

$$C_S / C_F = D_F$$
$$8,300 / 200 = 41.5$$

Calculate the final volume  $V_D$  required to prepare the **Standard #1** at 200 ng/mL

$$V_A * D_F = V_T$$
$$V_D = V_T - V_A$$

$$20 * 41.5 = 830 \mu\text{L}$$
$$V_D = 830 - 20 = 810 \mu\text{L}$$

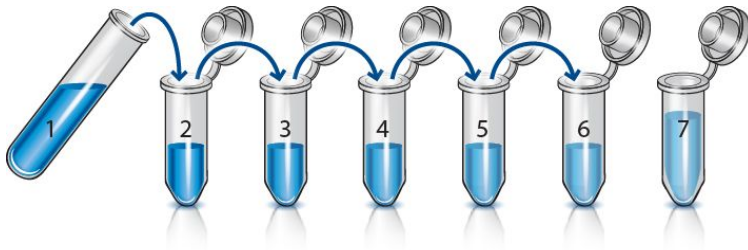
To tube #1, add 20  $\mu\text{L}$  of Dog Hemoglobin Calibrator to 810  $\mu\text{L}$  of 1X Diluent Solution to obtain a concentration of 200 ng/mL (**Standard #1**).

- 10.4 Add 300  $\mu\text{L}$  1X Diluent Solution into tube numbers 2-6.
- 10.5 Prepare **Standard #2** by adding 300  $\mu\text{L}$  **Standard #1** to tube #2. Mix thoroughly and gently.
- 10.6 Prepare **Standard #3** by adding 300  $\mu\text{L}$  from **Standard #2** to #2. Mix thoroughly and gently.
- 10.7 Using the table below as a guide to prepare further serial dilutions.
- 10.8 1X Diluent Solution serves as the zero standard (0 ng/mL).

# ASSAY PREPARATION

## Standard Dilution Preparation Table

Standard #	Volume to Dilute	Diluent ( $\mu\text{L}$ )	Total Volume ( $\mu\text{L}$ )	Final Conc. (ng/mL)
1	See step 10.3			200
2	300 $\mu\text{L}$ Std. 1	300	600	100
3	300 $\mu\text{L}$ Std. 2	300	600	50
4	300 $\mu\text{L}$ Std. 3	300	600	25
5	300 $\mu\text{L}$ Std. 4	300	600	12.5
6	300 $\mu\text{L}$ Std. 5	300	600	6.25
7 (Blank)	0	600	600	0.0



## 11. SAMPLE COLLECTION AND STORAGE

- 11.1 **Serum** – Blood should be collected by venipuncture. The serum should be separated from the cells after clot formation by centrifugation.
- 11.2 **Plasma** – 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.

- **Precautions**

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

- **Additives and Preservatives**

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

## 12. SAMPLE PREPARATION

- Please note that levels of the target protein may vary between different specimens. Optimal dilution factors for each sample must be determined by the investigator.
- The assay for quantification of Hemoglobin in samples requires that each test sample be diluted before use. For a single step determination, dilution of 1/10,000 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. **If unsure of sample level, a serial dilution with one or two representative samples before running the entire plate is highly recommended.**
- To prepare a 1/10,000 dilution of sample, transfer 5  $\mu$ L of sample to 495  $\mu$ L of 1X diluent. This gives you a 1/100 dilution. Next, dilute the 1/100 samples by transferring 5  $\mu$ L to 495  $\mu$ L of 1X diluent. You now have a 1/10,000 dilution of your sample. Mix thoroughly at each stage.

## 13. PLATE PREPARATION

- The 96 well plate strips included with this kit is supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused well plate strips should be returned to the plate packet and stored at 4°C.
- For each assay performed, a minimum of 2 wells must be used as blanks, omitting primary antibody from well additions.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

## **14. ASSAY PROCEDURE**

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**
  - 14.1 Pipette 100  $\mu$ L of each standard, including zero control, in duplicate, into the designated wells.
  - 14.2 Pipette 100  $\mu$ L of sample (in duplicate) into the designated wells.
  - 14.3 Incubate the microplate at room temperature for thirty ( $30 \pm 2$ ) minutes. Keep plate covered and level during incubation.
  - 14.4 Following incubation, aspirate the contents of the wells.
  - 14.5 Completely fill each well with appropriately diluted 1X Wash Buffer 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 gently striking the wells on absorbent paper to remove residual buffer. Repeat 3 times for a total of four washes.
  - 14.6 Pipette 100  $\mu$ L of appropriately diluted 100X Enzyme-Antibody Conjugate to each well. Incubate at room temperature for thirty ( $30 \pm 2$ ) minutes. Keep plate covered in the dark and level during incubation.
  - 14.7 Wash and blot the wells as described in 14.4 - 14.5.
  - 14.8 Pipette 100  $\mu$ L of TMB Substrate Solution into each well.
  - 14.9 Incubate in the dark at room temperature for precisely ten (10) minutes.
  - 14.10 After ten minutes, add 100  $\mu$ L of Stop Solution to each well.
  - 14.11 Immediately measure the absorbance of the plate at 450 nm using a microplate reader. Calibrate the plate reader to manufacturer's specifications, if necessary.

## ASSAY PROCEDURE

**Note:** *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*



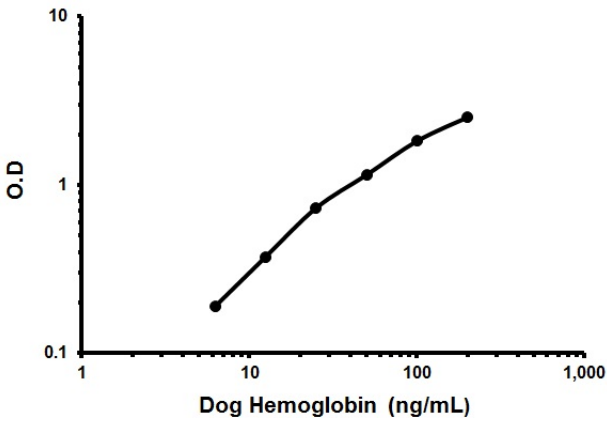
## 15. CALCULATIONS

- 15.1 Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.
- 15.2 Create a standard curve by plotting the blank control subtracted absorbance value for each standard concentration (y-axis) against the target protein concentration (x-axis). Individual absorbance values or average absorbance values can be plotted based on the graphing program used. Draw the best smooth curve through these points to construct the standard curve.

*Note:* Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four parameter curve fit (4PL) is typically the best choice however, other algorithms can be examined to see which can best fit the plotted values (e.g. linear, semi-log, log/log, 4 parameter logistic).

- 15.3 Determine the concentration of the target protein in the sample by interpolating the blank control subtracted absorbance values against the standard curve. Multiply the determined target concentration by the appropriate sample dilution factor as needed.
- 15.4 Samples generating absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest standard should be retested in a less dilute form.

16. TYPICAL DATA



**Figure 1:** Sample Standard Curve. A standard curve should be generated each time the test is performed.

Concentration (ng/mL)	Background Adjusted OD Value
6.25	0.191
12.5	0.375
25	0.731
50	1.149
100	1.824
200	2.519

## 17. TYPICAL SAMPLE VALUES

### SENSITIVITY -

Calculated minimum detectable dose = 3.107 ng/mL

### RECOVERY -

Control Serum Recovery = > 85%

### PRECISION –

	Intra-Assay	Inter-Assay
%CV	<10%	<10%

## 18. INTERFERENCES

These chemicals or biologicals will cause interferences in this assay causing compromised results or complete failure:

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

## 19. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipette performance
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation time; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
High %CV	Inaccurate pipetting	Check pipette performance
	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, ensure it is working properly.

20. NOTES





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