

ab157733 – Hemoglobin Rat ELISA Kit

Instructions for Use

For the quantitative measurement of hemoglobin in rat biological samples.

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

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1. BACKGROUND

Abcam's Hemoglobin rat ELISA kit is an *in vitro* enzyme-linked immunosorbent assay (ELISA) for the quantitative measurement of Hemoglobin in rat biological samples.

In this assay the hemoglobin present in samples reacts with the anti-hemoglobin antibodies which have been adsorbed to the surface of polystyrene microtiter 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 amount of enzyme bound in complex is measured by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies proportionately 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.

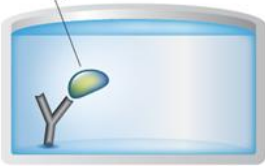
2. ASSAY SUMMARY

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 to each well used. 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. Immediately begin recording the color development

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 the components kit at +2-8°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)
Rat Hemoglobin ELISA Microplate	96 wells	2-8°C
Rat Hemoglobin Calibrator (Lyophilized)	1 vial	2-8°C
5X Diluent Concentrate	50 mL	2-8°C
20X Wash Buffer Concentrate	50 mL	2-8°C
100X HRP-conjugated anti-rat Hemoglobin antibody	150 µL	2-8°C
Chromogen Substrate Solution	12 mL	2-8°C
Stop Solution	12 mL	2-8°C

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 μ L to 200 μ L volumes.
- Adjustable 1-25 mL pipettes for reagent preparation.
- Timer
- Distilled or deionized water.
- Log-log graph paper or computer and software for ELISA data analysis.
- Tubes to prepare standard or sample dilutions.

7. LIMITATIONS

- This assay will perform as described only when the assay procedure is carefully followed 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 cape 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 1X Diluent Solution

The diluent solution is supplied as 5X Diluent Concentrate and must be diluted 1/5 with distilled or deionized water (1 part buffer concentrate, 4 parts dH₂O). The 1X Diluent Solution is stable for at least one week from the date of preparation and should be stored at 2-8°C.

9.2 1X Wash Buffer

The wash solution is supplied as 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. The 1X Wash Buffer is stable for at least one week from the date of preparation and can be stored at room temperature (16-25°C) or at 2-8°C.

9.3 100X HRP-conjugated anti-rat Hemoglobin antibody

Calculate the required amount of working conjugate solution for each microwell plate test strip by adding 10 µL HRP-conjugated anti-rat Hemoglobin antibody to 990 µL of 1X Diluent for each test strip to be used for testing. Mix uniformly, but gently. Avoid foaming. The working conjugate solution is stable for up to 1 hour when stored in the dark.

9.4 Rat Hemoglobin Calibrator

Reconstitute the Rat 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 µg/mL, where X is the amount on the vial (the reconstituted calibrator should be aliquoted and stored frozen if future use is intended).

9.5 Chromogen Substrate Solution

Ready to use as supplied.

9.6 Stop Solution

Ready to use as supplied.

10. STANDARD PREPARATIONS

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

10.1 The liquid Rat Hemoglobin Calibrator should be aliquoted and stored at - 20°C. Avoid multiple freeze-thaw cycles. **The calibrator is provided at the concentration stated on the vial.**

10.2 Label tubes numbers 1-6.

10.3 Prepare **Standard #1** by adding the appropriate volume of 1X Diluent Solution and Rat Hemoglobin Calibrator (derived below) to tube #1.

*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 Rat Hemoglobin Calibrator as labeled on the tube (as an example: 1,920 ng/mL)

C_F = Final concentration of Rat Hemoglobin Calibrator for the assay procedure (200 ng/mL)

V_A = Total volume of stock Rat Hemoglobin Calibrator to dilute e.g. 100 μ L)

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

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

D_F = Dilution factor

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

$$C_S/C_F = D_F$$
$$1,920 / 200 = 9.6$$

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

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

$$100 * 9.6 = 960 \mu\text{L}$$
$$V_D = 960 - 100 = 860 \mu\text{L}$$

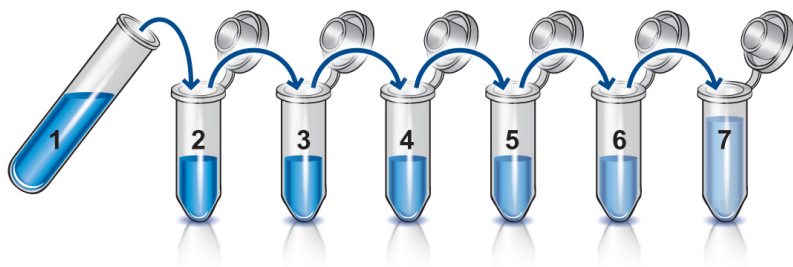
To tube #1, add 100 μL of Rat Hemoglobin Calibrator to 860 μL of 1X Diluent Solution to obtain a concentration of 200 ng/mL (**Standard #1**).

- 10.4 Prepare **Standard #1** by adding the appropriate volume of 1X Diluent Solution (like the example above) to tube #1. Add 100 μL of stock Rat Hemoglobin Calibrator to obtain a concentration at 200 ng/mL.
- 10.5 Add 300 μL 1X Diluent Solution into tube numbers 2-6.
- 10.6 Prepare **Standard #2** by adding 300 μL Standard #1 to tube #2. Mix thoroughly and gently.
- 10.7 Prepare **Standard #3** by adding 300 μL from **Standard #2** to #3. Mix thoroughly and gently.
- 10.8 Using the table below as a guide to prepare further serial dilutions.
- 10.9 1X Diluent Solution serves as the zero standard (0 ng/mL).

ASSAY PREPARATION

Standard Dilution Preparation Table

Standard #	Volume to Dilute (μL)	Diluent (μL)	Total Volume (μL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	See step 10.3				200
2	300	300	600	200	100
3	300	300	600	100	50
4	300	300	600	50	25
5	300	300	600	25	12.5
6	300	300	600	12.5	6.25
7	300	300	600	6.25	3.13



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** – 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.

- **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 azide contamination.

12. SAMPLE PREPARATION

General Sample information:

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 1/10,000 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 μL of sample to 495 μL of 1X diluent. This gives you a 1/100 dilution. Next, dilute the 1/100 samples by transferring 5 μL , to 495 μL 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)
- Well effects have not been observed with this assay. Contents of each well can be recorded on the template sheet included in the Resources section

14. ASSAY PROCEDURE

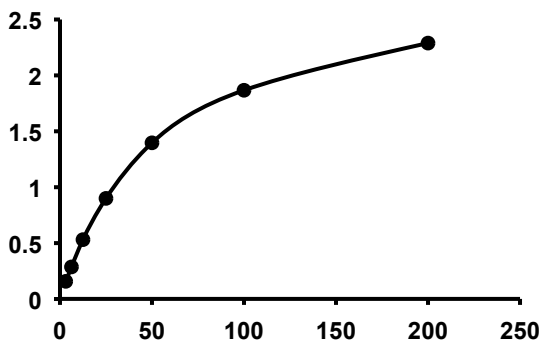
- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**
 - 14.1 Pipette 100 μ L of each standard, including zero control, in duplicate, into pre designated wells.
 - 14.2 Pipette 100 μ L of sample (in duplicate) into pre designated wells.
 - 14.3 Incubate the microtiter plate at room temperature for sixty (60 ± 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 μ L of appropriately diluted HRP-conjugated anti-Rat Hemoglobin Antibody to each well. Incubate at room temperature for thirty (30 ± 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 μ 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 μ L of Stop Solution to each well.
 - 14.11 Determine the absorbance (450 nm) of the contents of each well. Calibrate the plate reader to manufacturer's specifications.

15. CALCULATIONS

Average the duplicate standard reading for each standard, sample and control blank. Subtract the control blank from all mean readings. Plot the mean standard readings against their concentrations and draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A four parameter algorithm (4PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4-parameter logistic). Extrapolate protein concentrations for unknown and control samples from the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted in 1X Incubation Buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

16. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



Standard Curve Measurements	
Conc. (ng/mL)	Mean O.D.
6.25	0.357
12.5	0.666
25	0.936
50	1.456
100	1.791
200	2.144

17. TYPICAL SAMPLE VALUES

SENSITIVITY –

Calculated minimum detectable dose = 1.4284 ng/mL

RECOVERY –

Control Serum Recovery = > 85%

PRECISION –

	% CV
Inter-Assay	< 10%
Intra-Assay	< 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 pipettes
	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 times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store the reconstituted protein at -80°C, all other assay components 4°C. Keep substrate solution protected from light.

20. NOTES



Technical Support

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