Hemoglobin Colorimetric Assay Kit

Item No. 700540

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size
700541	Hemoglobin Sample Buffer (10X)	1 vial/10 ml
700542	Hemoglobin Detector (4X)	2 vials/10 ml
700543	Hemoglobin Standard	4 vials/1 ml
400014	96-Well Solid Plate (Colorimetric Assay)	2 plates
400012	96-Well Cover Sheet	2 covers

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3640

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored at 4°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance between 560-590 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Hemoglobin (Hb or Hgb) is a globular protein that carries oxygen from the lungs to bodily tissue where it releases oxygen for cell use and then returns carbon dioxide (CO_2) from the tissue to the lungs. Hemoglobin is primarily found in erythrocytes.[1] The family of human hemoglobin molecules is a set of closely related proteins made up of four globular protein subunits (globulin chains). Each subunit is comprised of a protein chain tightly bound to a heme group. The heme group contains a charged iron ion held within a tetrapyrrole ring, also referred to as a porphyrin ring.[1] It is this iron ion which reversibly binds oxygen through covalent bonding.[2]

Beyond binding and transporting oxygen, hemoglobin also binds CO_2, carbon monoxide (CO), and nitric oxide (NO). While CO_2 is also carried by hemoglobin, it does not share this binding site. Rather CO_2 binds to the protein chains of the structure, leaving the iron free to bind oxygen.[2] CO binds competitively to the heme group and will block oxygen binding, leading to hypoxia and, in severe cases, death. NO binds to the thiol groups of the globin protein structure to form an S-nitrosothiol which dissociates into free NO and thiol again, as the hemoglobin releases oxygen from its heme site.[3] It is hypothesized that as NO is released from the hemoglobin molecule, the vasodilatory effect of the NO may aid in oxygen transport into hypoxic tissues.[3]

In addition to the transport of various molecules to and from tissues, hemoglobin also plays an important role in maintaining the shape of the red blood cells. Abnormal hemoglobin structures can disrupt the shape of red blood cells and impede their function and flow through blood vessels. This is the underlying cause of sickle-cell anemia.[1] Additional disease states involving hemoglobin include thalassemia (lacking one or more globulin chain), bone and marrow diseases, kidney disease, and diabetes.[2]

About This Assay

Cayman's Hemoglobin Colorimetric Assay provides a quick, reliable method for determining total hemoglobin concentration in a variety of biological samples, including tissue homogenates and cell lysates. Cayman's Hemoglobin Assay utilizes an optimized detection reagent which is non-toxic and reports accurate measurements of total hemoglobin concentrations in a variety of samples. This is unlike the universally accepted reference method of hemoglobin determination, which uses potassium cyanide as a reagent and commonly under-estimates hemoglobin levels. In the presence of heme, the detection reagent selectively reacts with the heme molecule to create a product which absorbs between 560-590 nm. The intensity of the color is directly proportional to the total concentration of hemoglobin in the sample, based on the 4:1 molar ratio of heme to hemoglobin.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Hemoglobin Sample Buffer (10X) - (Item No. 700541)**

   The vial contains 10 ml of concentrated Sample Buffer (1M potassium phosphate, pH 7.4, containing 10 mM EDTA). Dilute the contents of the vial with 90 ml of HPLC-grade water. The diluted Sample Buffer (100 mM potassium phosphate, pH 7.4, containing 1 mM EDTA) is used for dilution of samples. When stored at 4°C, the solution is stable for six months.

2. **Hemoglobin Detector (4X) - (Item No. 700542)**

   Each vial contains 10 ml of concentrated Hemoglobin Detector. Dilute the contents of the vial with 30 ml of HPLC-grade water. This is enough detector to assay one plate. If assaying both plates, then prepare the other detector vial. When stored at 4°C, the diluted Hemoglobin Detector is stable for at least one month.

3. **Hemoglobin Standard - (Item No. 700543)**

   Each vial contains 1 ml of 500 µM Heme. It is ready to be used as supplied to prepare the standard curve. When stored at 4°C, the standard is stable for six months.

Sample Preparation

Plasma

1. Collect blood using an anticoagulant such as heparin or citrate.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C. Repeated freeze/thaw cycles should be avoided.
3. Plasma does not need to be diluted before assaying.

Serum

1. Collect blood without using an anticoagulant such as heparin or citrate.
2. Allow blood to clot for 30 minutes at 25°C.
3. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. Repeated freeze/thaw cycles should be avoided.
4. Serum does not need to be diluted before assaying.

Whole Blood

Normal hemoglobin concentration in human blood ranges from 12-18 g/dl.[4]

1. Collect blood using an anticoagulant such as heparin or citrate.
2. Store the blood at 4°C. If not assaying on the same day, freeze sample at -80°C.
3. Whole blood must be diluted with Sample Buffer before running in the assay. A dilution of ≥1:10 is typically required before assaying the sample.

Red Blood Cells

1. Collect blood using an anticoagulant such as heparin or citrate.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer. Pipette off the white buffy layer. Collect the red blood cells from the lower layer and store on ice until assaying.
3. If not assaying on the same day, freeze the sample at -80°C.
4. A total dilution of ≥1:10 is typically required before assaying the sample.

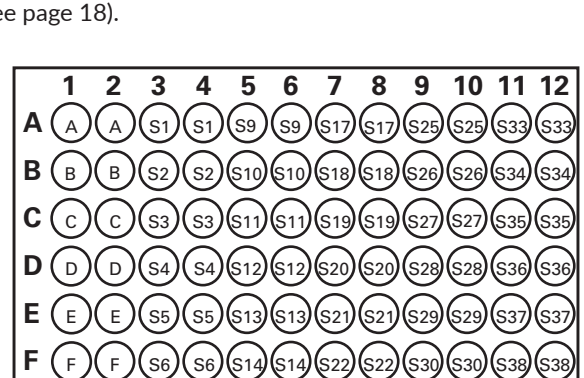
Tissue Homogenate

1. Prior to dissection, perfuse or rinse tissue with a phosphate buffered saline (PBS) solution, pH 7.4, containing 0.16 mg/ml heparin, to remove any extraneous red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of PBS solution, pH 7.4, containing 0.16 mg/ml heparin, per gram weight of tissue.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice. If not assaying on the same day, freeze the sample at -80°C.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of hemoglobin standards and samples to be measured in duplicate is given below in Figure 1. We suggest you record the contents of each well on the template sheet provided (see page 18).



A-H = Standards
S1-S40 = Sample wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 200 µl in all the wells.
- All reagents must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples be assayed at least in duplicate (triplicate is recommended).
- Twenty-four samples can be assayed in triplicate or forty in duplicate.
- The assay is performed at room temperature.
- Monitor the absorbance at 560-590 nm.

Standard Preparation

Take eight clean glass test tubes or polystyrene plastic tubes and label them A-H. Add the amount of Hemoglobin Standard and Hemoglobin Detector to each tube as described in Table 1.

Tube	500 µM Hemoglobin Standard (µl)	Hemoglobin Detector (µl)	Final Heme Concentation (µM)	Final Hemoglobin Concentation (g/dl)
A	0	500	0	0
B	10	490	10	0.016
C	25	475	25	0.040
D	50	450	50	0.080
E	100	400	100	0.160
F	150	350	150	0.240
G	200	300	200	0.320
H	250	250	250	0.400

Table 1. Preparation of standards

Performing the Assay

1. **Standard Wells -** add 200 µl of Hemoglobin Standard (tubes A-H) per well in the designated wells on the plate (see suggested plate configuration, Figure 1, on page 10).
2. **Sample Wells -** add 20 µl of sample to at least two wells per sample.
3. Add 180 µl of Hemoglobin Detector to each sample well. Do not add detector to standard wells.
4. Cover the plate with plate cover and incubate at room temperature for 15 minutes.
5. Remove the plate cover and read the absorbance at 560-590 nm. *NOTE: Make sure bubbles are removed before reading absorbance.*

ANALYSIS

Calculations

1. Calculate the average absorbance of each standard.
2. Subtract the average absorbance of standard A from itself and all other values (standards and samples). This is the corrected absorbance.
3. Plot the corrected absorbance of the standards (from step 2 above) as a function of the final concentration of Heme from Table 1. Either µM or g/dl values may be used to plot the results. See Figure 2, on page 15 , for a typical standard curve.
4. Calculate the hemoglobin concentration of the samples using the equation obtained from the linear regression of the standard curve substituting corrected absorbance values for each sample.

$$\text{Hemoglobin (g/dl)} = \left[\frac{\text{Corrected sample absorbance - (y-intercept)}}{\text{Slope}}\right] \times 10^{*} \times \text{Sample dilution}$$

*Accounts for dilution of sample in the well

Performance Characteristics

Sensitivity:

This limit of detection for this assay is approximately 3 µM or 0.005 g/dl.

Precision:

When a series of 71 hemoglobin measurements of the same serum sample were performed on the same day under the same experimental conditions, the intra-assay coefficient of variation was 6.4%. When a series of eight serum assays were performed on eight different days under the same experimental conditions, the inter-assay coefficient of variation was 5.8%.

Representative Hemoglobin Standard Curve

The standard curve presented here is an example of the data typically provided with this kit; however, your results will not be identical to these. You must run a new standard curve - do not use these to determine the values of your samples.

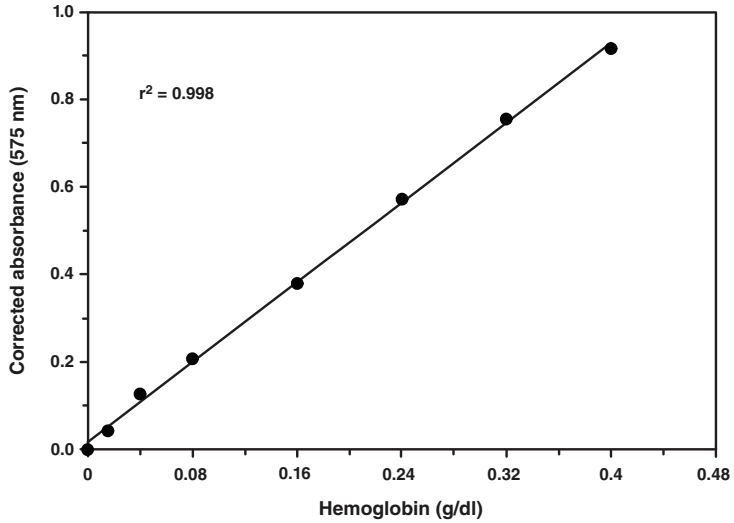


Figure 2. Hemoglobin standard curve

RESOURCES

Interferences

The following reagents were tested in the assay for interference in the assay:

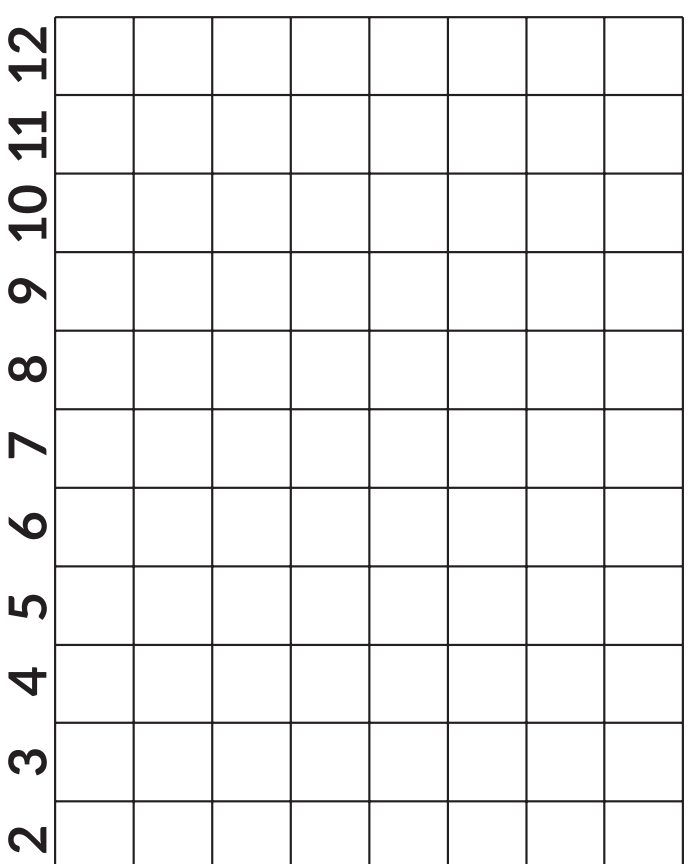
	Reagent	Will Interfere (Yes or No)
Buffers	Tris	No
	HEPES	No
	Phosphate	No
	Borate	No
Detergents	Polysorbate 20 (0.1%)	No
	Polysorbate 20 (1%)	No
	Triton X-100 (0.1%)	No
	Triton X-100 (1%)	No
Chelators	EDTA (1 mM)	No
	EGTA (1 mM)	No
Protease Inhibitors/ Enzymes	Trypsin (10 µg/ml)	No
	PMSF (200 µM)	No
	Leupeptin (10 µg/ml)	No
	Antipain (100 µg/ml)	No
	Chymostatin (10 µg/ml)	No
Solvents	Ethanol (5%)	No
	Methanol (5%)	No
	Dimethylsulfoxide (5%)	No
Others	BSA (1%)	No
	Glutathione (1 mM)	No
	Glycerol (5%)	No

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
Hemoglobin was not detected in the sample	Sample was too dilute	Re-assay the sample using a lower dilution
Absorbance of samples fell above the standard curve	The concentration of hemoglobin in the sample is too high	Dilute the sample with diluted Sample Buffer to fall within the range of the standard curve
The heme standard curve did not work	Either the heme standards were not diluted properly or the standard has deteriorated	Set up the standards according to Table 1 on page 12 and repeat the assay

References

1. Horton, H.R. Structure and function of biomolecules, Chapter 4, *in* Principles of Biochemistry. Horton, H.R., Moran, L.A., Ochs, R.S. *et al.*, editors, editors, 3, Neil Patterson Publishers, Englewood Cliffs, NJ, 4.30-4.41 (1993)
2. Schechter, Alan. Hemoglobin research and the origins of molecular medicine. *Blood* **112(10)**, 3927-3938 (2008).
3. Allen, B.W., Stamler, J.S., and Piantadosi, C.A. Hemoglobin, nitric oxide and molecular mechanisms of hypoxic vasodilation. *Trends Mol. Med.* **15(10)**, 452-460 (2009)
4. Lodemann, Peter. *et al*. Wrong molar hemoglobin reference values--a longstanding error that should be corrected. *Annals of Hemotology* **89**, 209 (2010).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.

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