ARBOR ASSAYS™

DetectX® Hemoglobin

High Sensitivity Colorimetric Detection Kit

2 Plate Kit – Catalog No. K013-HX1
10 Plate Kit – Catalog No. K013-HX5

Species Independent

Sample Types Tested:
Serum, EDTA Plasma, and Heparin Plasma

Please read this insert completely prior to using the product. For research use only.
Not for use in diagnostic procedures.
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SUPPLIED COMPONENTS & STORAGE

		K013-HX1	K013-HX5	Description
Clear 96-Well Plates	Quantity	2	2 x 5	Non-treated 96-well plates
	Catalog No.	X003-2EA	X003-5EA	
Hemoglobin Standard	Volume	90 µL	450 µL	Native human hemoglobin at 200 µg/mL in stabilizing solution
	Catalog No.	C246-90UL	C246-450UL	
Assay Buffer Concentrate 5X	Volume	28 mL	55 mL	5X concentrate that must be diluted
	Catalog No.	X067-28ML	X067-55ML	
DetectX® Hemoglobin Detection Reagent	Volume	2 x 11 mL	2 x 55 mL	Substrate solution
	Catalog No.	C248-11ML	C248-55ML	
Stop Solution	Volume	2 x 5 mL	2 x 25 mL	1M solution of hydrochloric acid **CAUSTIC**
	Catalog No.	X020-5ML	X020-25ML	

This kit should be stored at 4°C up to the expiration date on the kit label.

OTHER MATERIALS REQUIRED

- Distilled or deionized water
- Adjustable pipettes with disposable tips capable of dispensing 10 µL, 50 µL, and 100 µL. Repeater pipettes or multichannel pipettes with corresponding tips are also recommended.
- Glass or high-quality polypropylene test tubes for standard and sample preparation
- A plate reader capable of measuring optical density at 450 nm
- Software for converting optical density (OD) readings from the plate reader and carrying out a linear regression. Contact your plate reader manufacturer for details.

PRECAUTIONS

- Read this insert completely prior to using the product.
- This kit may not perform as described if any reagent or procedure is replaced or modified. Do not interchange reagents from different kit lots.
- Take appropriate safety precautions, such as: avoid breathing fumes, wear personal protective equipment (gloves, clothing, eye and face protection), and familiarize yourself with SDS documents.
    - https://www.arborassays.com/documentation/msds/K013-HX_MSDS.pdf
- The Hemoglobin Standard is derived from human blood. It has been extensively tested for viral contamination, but all human blood products should be treated as potentially infectious and adequate precautions taken.
- Ensure all buffers used for samples are azide free.
- **Take appropriate precautions when handling the Stop Solution, which is a caustic acid.**

BACKGROUND

Hemoglobin (Hgb) is an erythrocyte protein complex comprised of two sets of identical pairs of subunits, each binding an iron-porphyrin group commonly called heme[1]. Generally containing two alpha or alpha-like globulin chains, the remaining subunits may be beta, gamma, delta, or epsilon. Infants carry fetal hemoglobin that is replaced during the first year of life.

Heme binds and releases oxygen or carbon dioxide in response to partial pressure and changes in pH[1]. Free oxygen or carbon dioxide bound by one heme group facilitates subsequent binding by the other heme groups in each hemoglobin molecule[2]. Subtle changes in pH also regulate hemoglobin affinity for free gases, resulting in a high level of hemostatic control. Hemoglobin values are associated with a variety of conditions ranging from anemias (low Hgb), erythrocytosis (high Hgb), thalassemias (aberrant chain synthesis), and sickling disorders (abnormal complex shape)[1].

ASSAY PRINCIPLE

The DetectX® Hemoglobin High Sensitivity Detection Kit is designed to quantitatively measure all forms of hemoglobin present in plasma and serum. Please read this complete kit insert before performing the assay.

A human Hemoglobin Standard is provided to generate a standard curve for the assay and all samples should be read off the standard curve. Standards or diluted samples are pipetted into a clear microtiter plate and the ready-to-use DetectX® Hemoglobin Detection Reagent is added to each well. The plate is incubated for 30 minutes at room temperature. Stop solution is added at the end of 30 minutes and the plate is read at 450 nm. The concentration of the hemoglobin in the sample is calculated, after making suitable correction for dilution, using software available with most plate readers.

This assay has been validated for serum, EDTA plasma, and heparin plasma samples from multiple species.

REAGENT PREPARATION

Except for the reagents listed below, all kit components are ready for use.

Reagent	Preparation	Stability
1X Assay Buffer	Warm 5X Assay Buffer Concentrate to room temperature and mix thoroughly by inversion. Mix 1 volume 5X Assay Buffer Concentrate with 4 volumes deionized water.	1X Assay Buffer is stable for 3 months at 4°C

SAMPLE PREPARATION

Samples containing visible particulate should be centrifuged prior to use. Upon collection, all samples should be frozen rapidly and stored at -80˚C until testing.

Sample Type	Procedure
Serum and Plasma†*	• Collect sample with care to avoid hemolysis. • Prepare a minimum 20-fold dilution by adding 10 µL sample to 190 µL 1X Assay Buffer. • Samples may require further dilution with 1X Assay Buffer to fall within the standard curve.

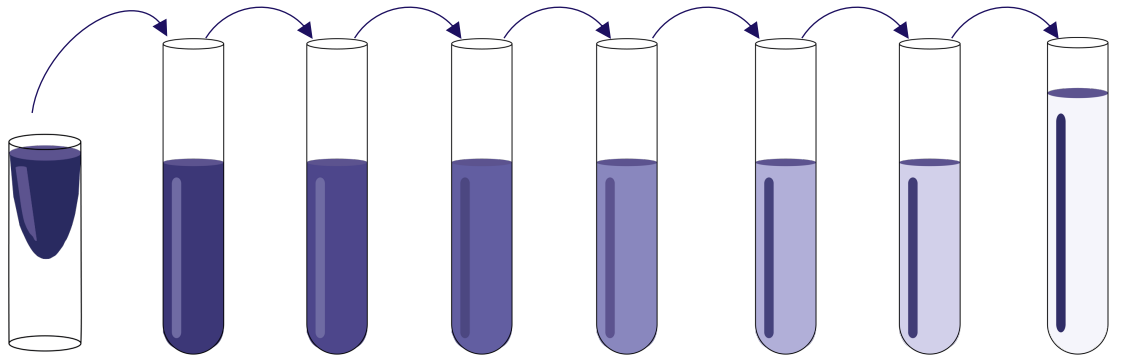
† For severely hemolyzed samples, the concentration of hemoglobin will be higher than expected due to damage of red blood cells during collection. Using these samples is not recommended.

* Samples with high lipid content may interfere with the measurement of hemoglobin.

⚠ **Use all samples within 2 hours of dilution or store diluted samples at -20ºC.**

STANDARD PREPARATION

1. Label tubes Standard 1 through Standard 7.
2. Add 90 µL 1X Assay Buffer to Standard 1 tube.
3. Add 50 µL 1X Assay Buffer to Standard 2 – 7 tubes.
4. Briefly vortex the Hemoglobin Standard stock solution, pulse spin in a microfuge, and add 10 µL to Standard 1 tube. Vortex thoroughly.
5. Transfer 50 µL of Standard 1 into Standard 2 tube to make a 2-fold dilution. Vortex thoroughly.
6. Transfer 50 µL of Standard 2 into Standard 3 tube to make a 2-fold dilution. Vortex thoroughly.
7. Continue serially diluting into the remaining tubes. This process and the final concentrations are summarized in the table below.



	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6	Standard 7
1X Assay Buffer (µL)	90	50	50	50	50	50	50
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Volume of Addition (µL)	10	50	50	50	50	50	50
Final Concentration (µg/mL)	20.0	10.0	5.0	2.5	1.3	0.63	0.31

⚠ **Use all Standards within 2 hours of dilution.**

ASSAY PROTOCOL

Before You Begin:

- **Room Temperature for this assay is defined as 22ºC – 24ºC.**
- **Ensure all reagents have been warmed to room temperature.**
- **Dilute samples as described in Sample Preparation.**
- **Run all standards and samples in duplicate.**
- Use the blank plate template on the back page of this booklet to design your plate layout and aid in proper sample and standard identification.
- Set plate parameters on the plate reader for a 96-well Corning CoStar 9017 plate. See ArborAssays.com for plate dimension data.

1. Add 10 µL of Samples or Standards into duplicate wells. Add 10 µL 1X Assay Buffer into duplicate Zero Standard wells.
2. Add 100 µL DetectX® Hemoglobin Detection Reagent to each well.
    - The solution will begin to turn blue.
3. Tap plate gently to mix. Incubate at room temperature for **30 minutes.**
4. Add 50 µL Stop Solution to each well.
    - The solution will begin to turn yellow.
5. Read the optical density at 450 nm within 10 minutes.

CALCULATION OF RESULTS

Follow the instructions below or use the online tool: https://myassays.com/assay.aspx?id=940

1. Use linear regression software to calculate the Hemoglobin concentration for each sample. Gather all raw data OD readings from each Sample and Standard, including the Zero Standard.

2. Average the duplicate OD readings for each Sample, Standard, and Zero Standard (Mean OD).

EXAMPLE:

Sample	Replicate 1 OD	Replicate 2 OD	Mean OD
Zero Standard	0.051	0.063	0.057
Standard 1	2.130	2.140	2.135
Sample 1	0.727	0.731	0.729

3. Subtract the Zero Standard Mean OD from the Mean OD for each Sample and Standard (Net OD).

EXAMPLE:

Sample	Mean OD	Zero Standard Mean OD	Net OD
Standard 1	2.135	0.057	2.078
Sample 1	0.729	0.057	0.672

4. Plot the standard curve with Net OD for the Standards on the y-axis and Hemoglobin concentration (µg/mL) on the x-axis. Perform a linear regression.

   Use the slope and Y-intercept of the regression line, together with the Net OD to calculate the Hemoglobin concentrations in diluted samples using the equation below. If diluted hemoglobin concentrations are outside of the range of the Standards, the Sample should be prepared again at a more appropriate dilution.

$$\text{Sample Hemoglobin Concentration (µg/mL)} = \frac{\text{(Net OD) – (Y-intercept)}}{\text{Slope}}$$

EXAMPLE:

Sample	Net OD	Sample Hemoglobin Concentration (µg/mL)
Sample 1	0.672	6.7

5. If the original sample was diluted, multiply the hemoglobin concentration by the sample dilution factor to determine the concentration of hemoglobin in the original sample.

EXAMPLE:

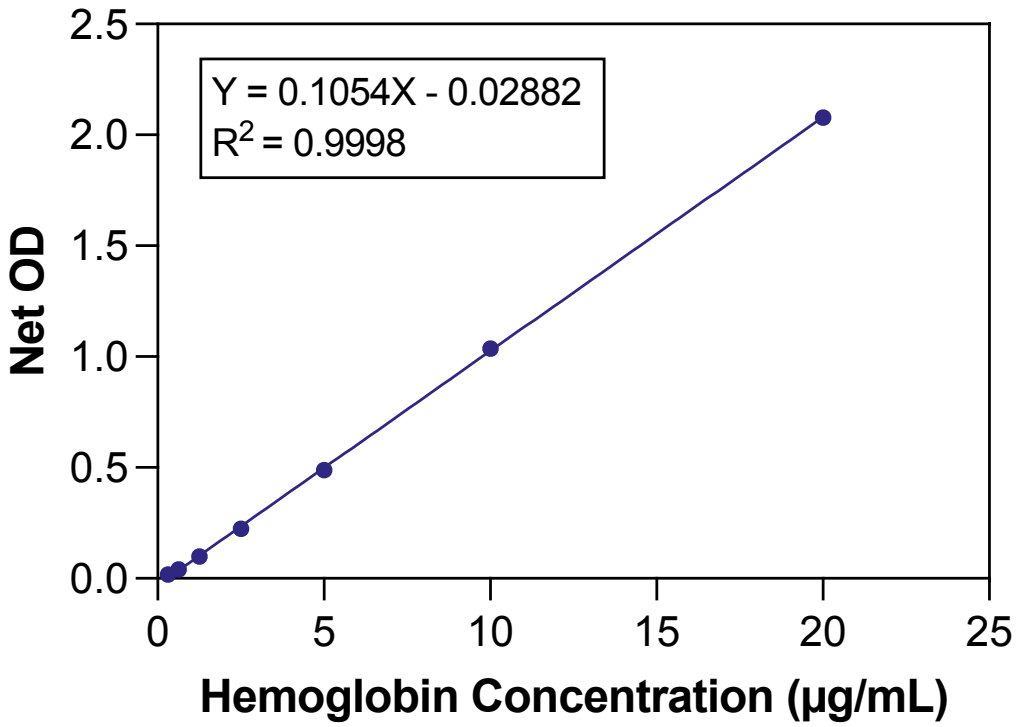
Sample	Sample Hemoglobin Concentration (µg/mL)	Sample Modification Factor	Original Sample Hemoglobin Concentration (µg/mL)
Sample 1	6.7	20x dilution	134

TYPICAL DATA

⚠ **Always run your own standard curve. This data should NOT be used to interpret results.**

Sample	Mean OD	Net OD	Hemoglobin Concentration (µg/mL)
Zero Standard	0.057	-	0
Standard 1	2.135	2.078	20.0
Standard 2	1.094	1.037	10.0
Standard 3	0.546	0.489	5.0
Standard 4	0.281	0.224	2.5
Standard 5	0.155	0.098	1.3
Standard 6	0.097	0.040	0.63
Standard 7	0.074	0.017	0.31
Sample 1	0.729	0.672	6.7
Sample 2	0.319	0.262	2.7

Typical Standard Curve



VALIDATION DATA

Sensitivity and Limit of Detection

Sensitivity was calculated by comparing the ODs for twenty wells run for each of the Zero Standard and Standard 7. The detection limit was determined at two standard deviations from the zero along the standard curve.

Sensitivity was determined as 0.042 µg/mL.

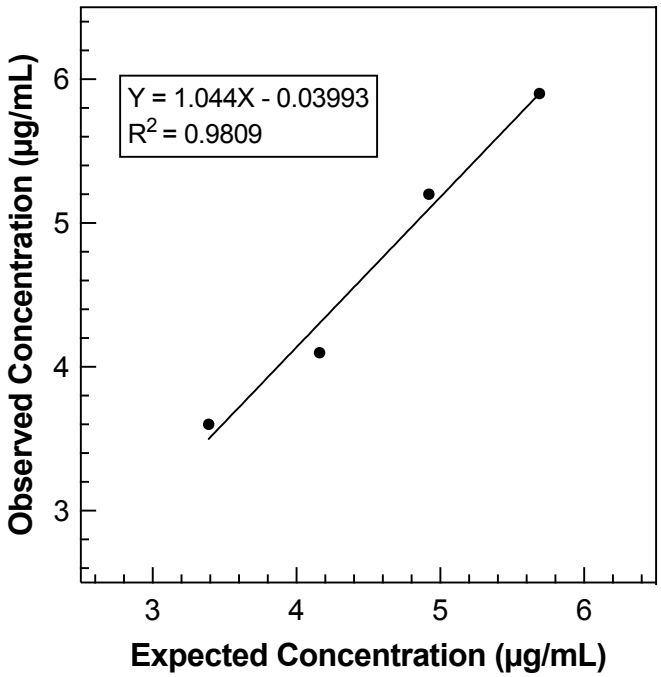
The Limit of Detection was determined in a similar manner by comparing the ODs for twenty wells run for each of the Zero Standard and a low concentration sample.

The Limit of Detection was determined as 0.023 µg/mL.

Linearity

Linearity was determined in rabbit serum by diluting two samples with known hemoglobin concentration with 1X Assay Buffer. One sample had a hemoglobin concentration of 2.6 µg/mL (low sample), and a second had a hemoglobin concentration of 6.5 µg/mL (high sample). The two samples were mixed in the ratios given below and the measured concentrations were compared to the expected values for each given ratio.

Low Sample	High Sample	Expected Concentration (µg/mL)	Observed Concentration (µg/mL)	% Recovery
80%	20%	3.4	3.6	104.9
60%	40%	4.2	4.1	99.6
40%	60%	4.9	5.2	104.8
20%	80%	5.7	5.9	103.9
			Mean Recovery	**103.3%**



Intra Assay and Inter Assay Precision

For intra assay precision, three serum samples were diluted in 1X Assay Buffer and 22 replicates were run in one assay. For inter assay precision, three serum samples were diluted in 1X Assay Buffer and duplicates of each sample were run in 20 assays over multiple days by four operators. The %CV represents the variation in concentration (not optical density) as determined using a standard curve.

	Intra Assay Precision		Inter Assay Precision	
Sample	Hemoglobin Concentration (µg/mL)	% CV	Hemoglobin Concentration (µg/mL)	% CV
1	6.5	1.8	6.5	3.8
2	4.4	1.6	4.3	4.6
3	2.7	2.8	2.6	4.8

SAMPLE VALUES

Five human serum and 7 human plasma samples, one of which was moderately hemolyzed, were diluted in 1X Assay Buffer and tested in the assay. The adjusted average concentration and sample range are shown below. Please refer to the Mayo Clinic for information on hemoglobin reference ranges[3].

Sample Type	Recommended Minimum Dilution	Adjusted Average Concentration (µg/mL)	Adjusted Average Concentration (µg/mL) Range
Serum	1:20	113.6	7.4 – 325.1
Plasma	1:10	136.0	13.2 – 713.6

INTERFERENCE

Bilirubin was evaluated at high and low concentrations of hemoglobin to evaluate its potential to interfere with the assay.

Interferent	Effect at High Hemoglobin Concentration	Effect at Low Hemoglobin Concentration
Bilirubin (0.5 mg/dL)	28.4% Decrease in Signal	18.9% Decrease in Signal
Bilirubin (0.05 mg/dL)	5.4% Decrease in Signal	3.7% Decrease in Signal

TROUBLESHOOTING

Issue	Possible Cause & Solution
Reagent Shortage	• Check under the cap for additional reagent. Pulse spin reagent containers to collect contents prior to opening when possible. • When using a multichannel pipette, return unused reagent to container for later use.
Erratic Values	• Prerinse pipet tips with desired reagent prior to aspirating the required volume. • Deliver volume with care to prevent splashing into adjacent wells.
High Background	• Reagent contamination during assay setup.
Low Signal	• Confirm tools, equipment, reagents, and containers used do not contain any trace of sodium azide. • Verify the plate reader wavelength is 450 nm.

CITATIONS

1. Tietz, N. W. (1986). Textbook of clinical chemistry. Philadelphia, PA: W. B. Saunders.
2. Manning, J. M., et al. (1998). Normal and abnormal protein subunit reactions. Journal of Biological Chemistry, 273(13), 19459–19362.
3. “Test ID: PLHBB Plasma Hemoglobin, Plasma.” MayoMedicalLaboratories.com. https://www.mayomedicallaboratories.com/test-catalog/clinical+and+interpretive/9096

RELATED PRODUCTS

Kits	Catalog No.
Acetylcholinesterase Fluorescent Activity Kit	K015-F1
Butyrylcholinesterase Fluorescent Activity Kit	K016-F1
Corticosterone Multi-Format ELISA Kits	K014-H1/H5
Cortisol ELISA Kits	K003-H1/H5
Cystatin C ELISA Kit	K012-H1
Glutathione Colorimetric Detection Kit	K006-H1
Glutathione Fluorescent Detection Kits	K006-F1/F5
Glutathione Reductase Activity Kit	K009-F1
Hemoglobin Colorimetric Detection Kit	K013-H1
Prostaglandin E2 Multi-Format ELISA Kits	K051-H1/H5
Retinol Binding Protein Multi-Format ELISA Kits	K062-H1/H5
Serum Creatinine Detection Kits	KB02-H1/H2

LIMITED WARRANTY

Arbor Assays warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

We must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if we are not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

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CONTACT INFORMATION

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OFFICIAL SUPPLIER TO ISWE

Arbor Assays and the International Society of Wildlife Endocrinology (ISWE) signed an exclusive agreement for Arbor Assays to supply ISWE members with assay kits and reagents for wildlife conservation research.

PLATE LAYOUT

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