Hemin Colorimetric Assay Kit

(Catalog #K672-100; 100 assays; Store kit at 4°C)

I. Introduction:

Free hemin results from the breakdown of hemin-containing proteins such as hemoglobin and myoglobin. It can be detected in various body fluids such as saliva, urine and CSF under various pathological states. Free hemin exists in cells at very minute concentrations (< 1µM ≈ 650 ng/ml) exerting regulatory functions such as repression of nonspecific δ-aminolevulinate synthase expression and induction of microsomal hemin oxygenase-1. Hemin can stimulate growth of oral bacteria involved in gingivitis and is an indicator of possible pathological conditions when found in the urine or feces. BioVision's Hemin Assay Kit utilizes peroxidase activity in the presence of hemin to provide a simple, exquisitely sensitive assay which causes the conversion of a colorless probe to a strongly colored (λ = 570 nm) compound. Trace amounts of Hemin can be quantified in the 5 - 160 pg (10 - 250 fmol) range.

II. Kit Contents:

Components	100 assays	Cap Code	Part Number
Hemin Assay Buffer	25 ml	WM	K672-100-1
Hemin Probe (in DMSO)	0.2 ml	Red	K672-100-2
Enzyme Mix	1 vial	Green	K672-100-3
Hemin Substrate	1 ml	Blue	K672-100-4
Hemin Standard (1 nmol; lyophilized)	1 vial	Yellow	K672-100-5

III. Reagent Preparation and Storage Conditions:

Probe: Warm at 37°C for 1 - 2 min to completely melt before use. Mix well, store at 4°C, protect from light and moisture. Use within two months.

Enzyme Mix: Dissolve in 0.5 ml Hemin Assay Buffer, mix well. Store at -20° C.

Hemin Substrate: Ready to use as supplied. Store at 4°C; Use within two months.

Hemin Standard: Dissolve with 100 µl DMSO to make a 10 µM solution. Store at 4°C; Use within two months.

IV. Hemin Assay Protocol:

1. **Standard Curve Preparations:** Immediately before use, dilute the 10 µM Hemin Standard to 100 nM by adding 10 µl of the Standard to 990 µl of Hemin Assay Buffer, mix well. Dilute further to 10 nM (= 10 fmol/µl) by adding 100 µl to 900 µl Hemin Assay buffer. Add 0, 4, 8, 12, 16, 20 µl into a series of wells. Adjust volume to 50 µl/well with Hemin Assay Buffer to generate 0, 40, 80, 120, 160, 200 fmol/well of the Hemin Standard.

2. **Sample Preparations:** Depending upon the hemin content, samples should be diluted typically 100 to 10,000 fold and added at about 1 - 10 µl of diluted sample per well. Samples can be assayed without any prior treatment*. Hemin concentration in samples may have a wide range. For different sample types, we suggest using ~ 0.04 µl serum sample, ~ 50 µg of feces, ~ 1 - 5000 cultured cells or ~ 0.05 µl urine. Place diluted samples directly in wells and adjust well volumes to 50 µl with Hemin Assay Buffer in a 96-well plate. We suggest using several doses of your sample to ensure the readings are within the standard curve range.

   *The presence of hemoproteins may interfere with the assay although in our experience, the very high dilution factor reduces the concentration of any such proteins to undetectable levels. You may do a sample background control without the Enzyme Mix in the reaction, then subtract the sample background from your sample readings.

3. **Reaction Mix Preparation: NOTE: The proper order of addition of the following components is critical.** Immediately before use, mix enough reagent for the number of assays performed. For each well, prepare a 50 µl Reaction Mix containing the following components **in the following order**.

   1. Enzyme Mix 3 µl
   2. Hemin Substrate 2 µl

   Incubate for 2 minutes at room temperature before adding the following components:

   3. Assay Buffer 43 µl
   4. Probe 2 µl

4. Add 50 µl of the Reaction Mix to each well containing the Hemin Standard or test samples, mix well.

5. Incubate the reaction for 10-30 min at room temperature, protect from light. As this is an enzyme activity assay, it is important to incubate and measure your samples at the same time and under exactly the same conditions as the standards. The 10-30 minute incubation time has been selected as the best compromise between linearity, speed and sensitivity. It is advantageous to read the assay in kinetic mode (as shown below), observing the color development as it proceeds, using measurement data in the range of 0.7-1.3 OD for the highest standard (200 fmol).

6. Measure the OD at 570 nm.

7. **Calculation:** Correct background by subtracting the value derived from the 0 Hemin control from all sample and standard readings (Note: The background reading may be significant and must be subtracted from sample readings). Plot standard curve pmol/well vs. OD 570 nm readings. Then apply the sample readings to the standard curve to get Hemin amount in the sample wells (Hy). Calculate the Hemin concentrations in the test samples as follows:

   C (fmol/µl or nM) = Hy/Sv x Ds

   Where: Hy is the amount of Hemin (fmol) of your sample from standard curve.
   Sv is the sample volume (µl) added into the sample well.
   Ds is the dilution factor of the sample, i.e. 100 or 10,000

   Hemin molecular weight: 652. Hemin concentration in your sample can be expressed as pmol/ml, ng/ml, µg/dL or µM (µmol/liter); 1 µM = 1 nmol/ml = 652 ng/ml.

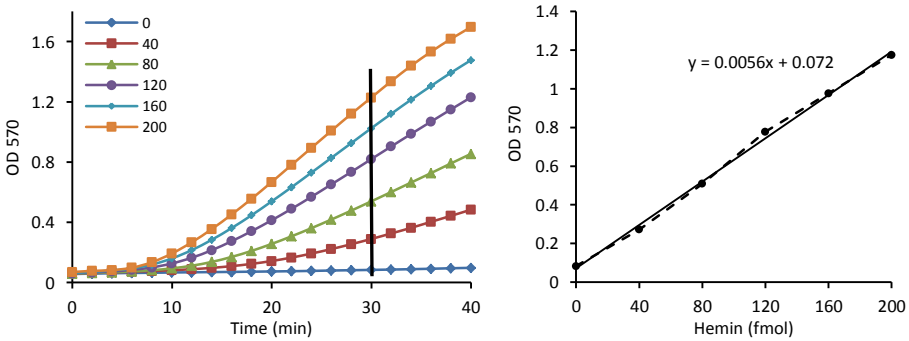

Fig. 1 A: Time course of development of color and B: Standard Curve at 30 minutes for Hemin standard as performed according to this protocol

RELATED PRODUCTS:

Lactate Assay Kit	Glutathione Assay Kit
Ascorbic acid Assay Kit	Free Fatty Acid Assay Kit
NAD(P)/NAD(P)H Assay Kit	ATP/ADP Assay Kit
Cell Proliferation Assay Kit	Cytotoxicity Assay Kit

GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	• Use of ice-cold assay buffer	• Assay buffer must be at room temperature
	• Omission of a step in the protocol	• Refer and follow the data sheet precisely
	• Plate read at incorrect wavelength	• Check the wavelength in the data sheet and the filter settings of the instrument
	• Use of a different 96-well plate	• Fluorescence: Black plates (clear bottoms) ; Luminescence: White plates ; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type	• Refer data sheet for details about incompatible samples
	• Samples prepared in a different buffer	• Use the assay buffer provided in the kit or refer data sheet for instructions
	• Samples were not deproteinized (if indicated in datasheet)	• Use the 10 kDa spin cut-off filter or PCA precipitation as indicated
	• Cell/ tissue samples were not completely homogenized	• Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope
	• Samples used after multiple free-thaw cycles	• Aliquot and freeze samples if needed to use multiple times
	• Presence of interfering substance in the sample	• Troubleshoot if needed, deproteinize samples
	• Use of old or inappropriately stored samples	• Use fresh samples or store at correct temperatures till use
Lower/ Higher readings in Samples and Standards	• Improperly thawed components	• Thaw all components completely and mix gently before use
	• Use of expired kit or improperly stored reagents	• Always check the expiry date and store the components appropriately
	• Allowing the reagents to sit for extended times on ice	• Always thaw and prepare fresh reaction mix before use
	• Incorrect incubation times or temperatures	• Refer datasheet & verify correct incubation times and temperatures
	• Incorrect volumes used	• Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	• Use of partially thawed components	• Thaw and resuspend all components before preparing the reaction mix
	• Pipetting errors in the standard	• Avoid pipetting small volumes
	• Pipetting errors in the reaction mix	• Prepare a master reaction mix whenever possible
	• Air bubbles formed in well	• Pipette gently against the wall of the tubes
	• Standard stock is at an incorrect concentration	• Always refer the dilutions in the data sheet
	• Calculation errors	• Recheck calculations after referring the data sheet
	• Substituting reagents from older kits/ lots	• Use fresh components from the same kit
Unanticipated results	• Measured at incorrect wavelength	• Check the equipment and the filter setting
	• Samples contain interfering substances	• Troubleshoot if it interferes with the kit
	• Use of incompatible sample type	• Refer data sheet to check if sample is compatible with the kit or optimization is needed
	• Sample readings above/below the linear range	• Concentrate/ Dilute sample so as to be in the linear range
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		