



Hemoglobin ELISA

For the quantitative determination of hemoglobin in human stool.

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number: 30-HEMHU-E01

Size: 96 Wells

Version: 2022-02-22 – ALPCO 4.0

INTENDED USE

The Hemoglobin ELISA is intended for the quantitative determination of hemoglobin in human stool samples. For research use only. Not for use in diagnostic procedures.

PRINCIPLE OF THE ASSAY

This ELISA is used for quantitative determination of hemoglobin in stool. In a first incubation step, the hemoglobin in the sample is bound to anti-hemoglobin antibodies (in excess), which are immobilized on the surface of the microtiter wells. To remove all unbound substances, a washing step is carried out. In a second incubation step an anti-hemoglobin peroxidase labeled antibody is added. After another washing step, to remove all unbound substances, the solid phase is incubated with the substrate, tetramethylbenzidine (TMB). An acidic solution is then added to stop the reaction. The color converts from blue to yellow. The intensity of the yellow color is directly proportional to the concentration of hemoglobin in the sample. A curve of the absorbance unit (optical density, OD) vs. concentration is generated, using the values observed from the standards. The hemoglobin present in the samples is determined directly from this curve.

MATERIALS SUPPLIED

Component	Quantity	Preparation
Hemoglobin Microplate (96 wells)	12 x 8 strips	Ready to use
Standards (1-5) (0, 0.67, 3.3, 10, 50 µg/g)	2 x 5 vials	Lyophilized
Control Levels 1 and 2 (see Certificate of analysis for range)	2 vials each	Lyophilized
Wash Buffer Concentrate	2 x 100 mL	10X
Extraction Buffer Concentrate	100 mL	2.5X
Conjugate (mouse anti-human HB, peroxidase-labeled)	15 mL	Ready to use
Sample Dilution Buffer	2 x 15 mL	Ready to use
TMB Substrate	15 mL	Ready to use
Stop Solution	15 mL	Ready to use

MATERIALS REQUIRED BUT NOT SUPPLIED

- Precision pipettes for dispensing 10 to 1000 µL (with disposable tips)
- Repeating or multi-channel pipette for dispensing up to 1000 µL
- Volumetric containers and pipettes for reagent preparation
- Distilled/Deionized water for reagent preparation
- Microplate washer or wash bottle
- Microplate reader
- Centrifuge (3,000 x g) for sample preparation
- Vortex for sample preparation
- Laboratory Balance for sample preparation
- Foil to cover the microplate
- Timer

PRECAUTIONS

1. Human materials used in kit components were tested and found to be negative for HIV (Human Immunodeficiency Virus), HBV (Hepatitis B Virus), and HCV (Hepatitis C Virus). However, for safety reasons, all reagents should be treated as potentially infectious.

2. Kit reagents contain sodium azide or Proclin as bactericides. Sodium azide and Proclin are hazardous to health and the environment. Substrates for the enzymatic color reactions may also cause skin and/or respiratory irritation. Any contact with the substances must be avoided. Further safety information can be found in the safety data sheet, which is available from ALPCO upon request.
3. The 10x Wash Buffer concentrate (WASHBUF) contains surfactants which may cause severe eye irritation in case of eye contact.
Warning: Causes serious eye irritation. IF IN EYES: Rinse carefully with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If eye irritation persists, get medical advice/attention.
4. The stop solution consists of diluted sulfuric acid, a strong acid. Although diluted, it still must be handled with care. It can cause burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped out immediately with copious quantities of water. Do not breathe vapor and avoid inhalation.

STORAGE CONDITIONS

The kit should be stored at 2-8°C. With proper storage, the unopened kit is stable until the expiry date.

SAMPLE HANDLING

Stool samples are appropriate for use in this assay.

Due to the degradation of hemoglobin at room temperature, which can amount to 50% per day, **raw stool** samples should be shipped frozen. If shipment at either -20°C or cooler is not possible, the samples should be shipped overnight. This will still reduce the sensitivity.

The sample stability is as follows:

Raw stool can be stored for 1 month at -20°C (Thomas et al., 1998).

Stool extract is stable at room temperature (15-30°C), 2–8°C, as well as at -20°C for seven days. Avoid more than three freeze-thaw cycles.

Extraction of the Stool Sample: Diluted extraction buffer is used as a sample extraction buffer. It is recommended to perform the following sample preparation:

1. The raw stool sample must be thawed. Particularly heterogeneous samples should be mechanically homogenized using an applicator, inoculation loop, or similar device.
2. Fill an empty sample tube with 1.5 mL of prepared extraction buffer (diluted 1:2.5) before addition of 15 mg of raw stool sample. Important: Allow the buffer to reach room temperature. If using the EZ Extraction Device, the device is pre-filled and does not require any additional extraction buffer.
3. For device use, unscrew the tube (yellow part of cap) to open. Insert yellow dipstick into sample. The lower part of the dipstick has notches that must be covered completely with stool after inserting it into the sample. Place dipstick back into the tube. When putting the stick back into the tube, excess material will be stripped off; leaving 15 mg of sample to be diluted. Screw tightly to close the tube.
4. Shake the tube well until no stool sample remains in the notches. Important: Make sure that it is a maximally homogenous suspension after shaking. Especially with more solid samples, soaking the sample in the tube with buffer for approximately 10 minutes improves the result.
5. Allow sample to stand for approximately 10 minutes until sediment has settled. Floating material like shells of grains can be neglected.

- Carefully unscrew the complete cap of the tube including the blue ring plus the dipstick. Discard cap and dipstick. Make sure that the sediment will not be dispersed again. This will result in a dilution of 1:100 (Dilution I).

The sample suspension is now ready for use.

REAGENT PREPARATION

All reagents must be equilibrated to room temperature prior to preparation and subsequent use in the assay. Prepare enough reagents for only the number of strips used. The kit can be used up to 4 times within the expiry date stated on the label. Reagents with a volume less than 100 µL should be centrifuged before use to avoid loss of volume.

Controls (Levels 1 and 2) and Standards are provided in a lyophilized form. Reconstitute in 500 µL of distilled or deionized water. Close the vial with the rubber stopper and cap, gently swirl the vial, and allow it to stand for 10 minutes prior to use. The contents of the vial should be in solution with no visible particulates. Lyophilized controls and standards are stable at 2-8°C until the expiry date stated on the label. Reconstituted controls and standards can be stored at 2-8°C for four weeks.

Wash Buffer Concentrate (10X) must be diluted with distilled or deionized water 1:10 before use (100 mL WASHBUF + 900 mL distilled water) and mixed well. Crystals could occur due to high salt concentration in the stock solutions. Before dilution, the crystals must be dissolved at room temperature or in a water bath at 37°C. The Wash Buffer concentrate is stable at 2-8°C until the expiry date stated on the label. Diluted Wash Buffer can be stored in a closed flask at 2-8°C for one month.

Extraction Buffer Concentrate (2.5X) must be diluted or deionized with distilled water 1:2.5 before use (100 mL Extraction Buffer + 150 mL distilled water) and mixed well. Crystals could occur due to high salt concentration in the stock solutions. Before dilution, the crystals must be dissolved at 37°C in a water bath. The Extraction Buffer concentrate is stable at 2-8°C until the expiry date stated on the label. Prepared extraction buffer (1:2.5 diluted) can be stored in a closed flask at 2-8°C for four months.

All other test reagents are ready to use. Test reagents are stable until the expiry date (see label of test package) when stored at 2-8°C.

QUALITY CONTROL

Control samples should be analyzed with each run. Results generated from the analysis of control samples should be evaluated for acceptability using appropriate statistical methods. The results for the samples may not be valid if within the same assay one or more values of the quality control sample are outside the acceptable limits.

ASSAY PROCEDURE

All reagents and microplate strips (while sealed in foil pouch) should be equilibrated to room temperature (15-30°C) prior to use. Gently mix all reagents before use. A standard curve must be performed with each assay run and with each microplate if more than one is used at a time. All standards, controls, and samples should be run in duplicate.

Bring frozen sample suspensions to room temperature (15-30°C) and then vortex. Allow the sample to stand for ~10 minutes until sediment has settled before using the supernatant in the assay. Take as many microtiter strips as needed from the kit. Store unused strips together with the desiccant bag in the closed aluminum packaging at 2-8°C. Strips are stable until the expiry date stated on the label.

For automated ELISA processors the given protocol may need to be adjusted according to the specific features of the respective automated platform. For further details please contact ALPCO.

1. Bring all reagents and samples to room temperature (15–30°C) and mix well.
2. Wash the pre-coated microtiter plate 5 times with 250 µL 1X working wash buffer before use. After the final washing step, remove the residual wash buffer by firmly tapping the plate on absorbent paper.
3. Add 50 µL of Sample Dilution Buffer to each well.
4. Add 50 µL of standards/controls/extract samples into respective wells. See *Reagent Preparation* for standard and control reconstitution instructions. A suggested plate map is included.
5. Cover plate tightly and incubate for 1 hour at room temperature (15–30°C).
6. Discard the contents of each well. Wash each well 5 times with 250 µL of wash buffer. After the final washing step, remove the residual wash buffer by firmly tapping the plate on absorbent paper.
7. Add 100 µL of conjugate into each well.
8. Cover plate tightly and incubate for 1 hour at room temperature (15–30°C).
9. Discard the contents of each well. Wash each well 5x with 250 µL of 1X working wash buffer. After the final washing step, remove the residual wash buffer by firmly tapping the plate on absorbent paper.
10. Add 100 µL of TMB substrate into each well.
11. Incubate for 10–20 minutes at room temperature (15–30°C) in the dark.*
12. Add 100 µL of stop solution into each well, mix thoroughly.
13. Determine absorption immediately with an ELISA reader at 450nm against 620nm (or 690nm) as a reference. If no reference wavelength is available, read only at 450nm. If the extinction of the highest standard exceeds the range of the photometer, absorption must be measured immediately at 405nm against 620nm as a reference.

* The intensity of the color change is temperature sensitive. It is recommended to observe the color change and to stop the reaction upon good differentiation.

CALCULATION OF RESULTS

The following algorithms can be used alternatively to calculate the results. It is recommended to use the 4-parameter algorithm.

1. 4-parameter algorithm: It is recommended to use a linear ordinate for the optical density and a logarithmic abscissa for the concentration. When using a logarithmic abscissa, the zero calibrator must be specified with a value less than 1 (e.g. 0.001).
2. Point-to-point calculation: It is recommended to use a linear ordinate for the optical density and a linear abscissa for the concentration.

- Spline algorithm: It is recommended to use a linear ordinate for the optical density and a linear abscissa for the concentration.

The plausibility of the pairs of values should be analyzed before the automatic evaluation of the results. If this option is not available with the program used, a comparison of the paired values should be done manually.

Stool Samples

Since the sample dilution is already considered in the calibration curve, the dilution factor is 1. In case another dilution factor has been used, multiply the obtained result with the additional dilution factor used.

LIMITATIONS

Samples with concentrations above the measurement range (see definition below) must be further diluted and re-assayed. Please consider this higher dilution when calculating the results. Samples with concentrations lower than the measurement range cannot be clearly quantified.

The upper limit of the measurement range can be calculated as:
 highest concentration of the standard curve × sample dilution factor to be used

The lower limit of the measurement range can be calculated as:
 Limit of Quantitation (LoQ) × sample dilution factor to be used

PERFORMANCE CHARACTERISTICS

Analytical Sensitivity

The following values have been estimated based on the concentrations of the standard without considering possibly used sample dilution factors.

Limit of blank, LoB: 0.09 µg/g
 Limit of detection, LoD 0.15 µg/g
 Limit of quantitation, LoQ 0.18 µg/g

Accuracy-Precision

Repeatability (intra-assay); n = 42

The repeatability was assessed with 2 stool samples under constant parameters (same operator, measurement system, day, and kit lot).

	Sample 1	Sample 2
Mean	3.99 µg/g	7 µg/g
CV (%)	4.8	2.6
n	42	42

Reproducibility (inter-assay); n = 68

	Sample 1	Sample 2
Mean	0.87 µg/g	3.51 µg/g
CV (%)	8.6	4.8
n	68	68

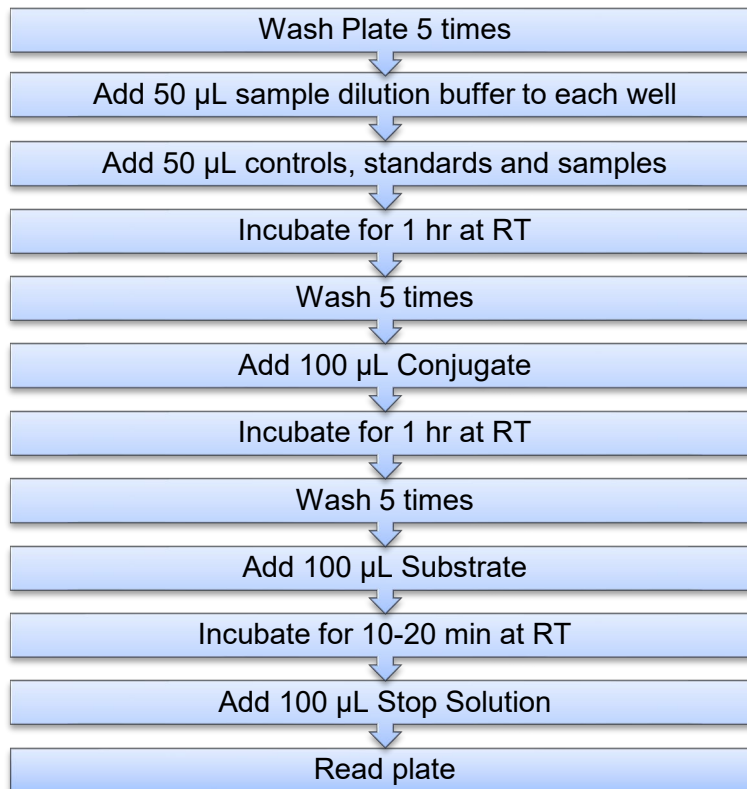
TECHNICAL HINTS

- Do not interchange different lot numbers of any kit component within the same assay. Furthermore, it is not recommended to assemble wells of different microtiter plates for analysis, even if they are of the same batch.
- Control samples should be analyzed with each run.
- Reagents should not be used beyond the expiration date stated on kit label.
- Substrate solution should remain colorless until use.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Avoid foaming when mixing reagents.
- Do not mix plugs and caps from different reagents.
- The assay should always be performed according to the enclosed manual.

GENERAL NOTES ON THE ASSAY AND ASSAY PROCEDURE

- Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which is not coordinated with the producer, may influence the results of the test. ALPCO can therefore not be held responsible for any damage resulting from incorrect use.
- Warranty claims and complaints regarding deficiencies must be logged within 14 days after receipt of the product.

SHORT ASSAY PROTOCOL



Total Time = 2 hours 20 minutes

SUGGESTED PLATE LAYOUT

Below is a suggested plate layout for running standards, controls, and up to 41 samples in duplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std1	Std 1	2	2	10	10	18	18	26	26	34	34
B	Std 2	Std 2	3	3	11	11	19	19	27	27	35	35
C	Std 3	Std 3	4	4	12	12	20	20	28	28	36	36
D	Std 4	Std 4	5	5	13	13	21	21	29	29	37	37
E	Std 5	Std 5	6	6	14	14	22	22	30	30	38	38
F	Ctrl 1	Ctrl 1	7	7	15	15	23	23	31	31	39	39
G	Ctrl 2	Ctrl 2	8	8	16	16	24	24	32	32	40	40
H	1	1	9	9	17	17	25	25	33	33	41	41

Std = Standard; Ctrl = Control; Numbered wells = Samples