Human Fetal Hemoglobin ELISA Quantitation Set

Cat. No. E80-136
Lot No. E80-136-22

Components Supplied

- Affinity purified Sheep anti-Human Fetal Hemoglobin Coating Antibody A80-136A-13, 1 ml at 1 mg/ml (1:100)
- Human Hemoglobin Calibrator, RC80-135-5, 0.1 ml (0.5 mg/ml Hemoglobin F)
- HRP Conjugated Sheep anti-Human Fetal Hemoglobin Detection Antibody A80-136P-22, 0.1 ml at 1 mg/ml (1:100,000)

Introduction

Enzyme linked immunosorbent assay (ELISA) for the detection of Human Fetal Hemoglobin in serum. Other biological fluids that contain Human Fetal Hemoglobin, such as plasma, urine, feces, saliva, may be suitable samples. The Set contains sufficient components to perform 1000 single well assays.

The set performance has been optimized for the stated protocol using the materials listed and standard dilutions from 400 – 6.25 ng/ml of Human Fetal Hemoglobin. *For alternative assay conditions, the operator must determine appropriate dilutions of reagents.*

Country of Origin: USA

Procedure Overview

1. Add 100 µl of diluted coating antibody to each well.
   Note: Run each standard or sample in duplicate.
2. Incubate at room temperature (20-25°C) for 1 hour.
3. Wash plate FIVE times.
4. Add 200 µl of Blocking Solution to each well.
5. Incubate at room temperature for 30 minutes.
6. Wash plate FIVE times.
7. Add 100 µl of standard or sample to well.
8. Incubate at room temperature for 1 hour.
9. Wash plate FIVE times.
10. Add 100 µl of diluted HRP detection antibody to each well.
11. Incubate at room temperature for 1 hour.
12. Wash plate FIVE times.
13. Add 100 µl of TMB Substrate Solution to each well.
14. Develop the plate in the dark at room temperature for 15 minutes.
15. Stop reaction by adding 100 µl of Stop Solution to each well.
16. Measure absorbance on a plate reader at 450 nm.

Plates, Buffers and Substrate not provided

- 96-well plate (Cat. No. E105)
- ELISA Coating Buffer (Cat. No. E107)
- ELISA Wash Solution (Cat. No. E106)
- ELISA Blocking Buffer (Cat. No. E104)
- Sample/Conjugate Diluent (ELISA Blocking Buffer + Tween 20)
- 10% Tween 20 (Cat. No. E108)
- Enzyme Substrate, TMB (Cat. No. E102)
- ELISA Stop Solution (Cat. No. E115)

The above products may be bought individually or bought together in the ELISA Starter Accessory Kit (Cat. No. E101). ELISA Stop Solution (Cat. No. E115) is not included in the kit and is sold separately. The E101 contains 10 x 96-well plates.

Buffers may be prepared in your lab according to the formulations specified under Buffer Preparation of this protocol.

Additional Materials Required

- Ultrapure water
- Precision pipettors, with disposable plastic tips
- Polypropylene, polyethylene or glass tubes to prepare standard and samples
- Containers to prepare buffers
- An aspiration device or an automated 96-well plate washer
- Disposable reagent reservoirs
- A standard microtiter plate reader for measuring absorbance at 450 nm

Precautions

- Store all reagents at 2-8ºC. Do not freeze reagents.
- All reagents must be at room temperature (20-25°C) before use.
- Vigorous plate washing is essential.
- Use new disposable pipette tips for each transfer to avoid cross-contamination.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can easily become contaminated thereby causing assay variability.
- Take care not to contaminate the TMB Solution. Do not expose TMB Substrate solution to glass, foil, or metal. If the solution is blue before use, DO NOT USE IT.

Buffer Preparation

Prepare the following buffers from the ELISA Starter Accessory Kit (E101) or your lab:

- ELISA Coating Buffer, 0.05 M Carbonate-Bicarbonate, pH 9.6
- ELISA Wash Solution, 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0
- ELISA Blocking Solution, 50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0
- Sample/Conjugate Diluent, 50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20
- Enzyme Substrate, TMB
- ELISA Stop Solution, 0.18 M H_2SO_4

Sample, Reagent, and Standard Handling and Preparation

Sample Handling

- Serum may be tested in this ELISA. Other fluids containing Human Fetal Hemoglobin may be tested but interpretation is subject to researcher.
- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions when handling and disposing of infectious agents.
- 100 µl of sample or standard is required per well.
- Samples must be assayed in duplicate each time the assay is performed.
- Store samples to be assayed within 24 hours at 2-8ºC. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles when storing samples.
- If particulate is present in samples, centrifuge prior to analysis.
- If samples are clotted, grossly hemolyzed, lipemic, or the integrity of the sample is of concern, make a note on the Plate Template and interpret results with caution.

Sample Dilution

- Dilute the samples, based on the expected concentration of the analyte, to fall within the concentration range of the standards.

Standard Dilution

- Standard should be treated as a biological material and universal precautions should be followed. Follow the recommended dilutions in the table provided under Standards and Samples.

PROCEDURE

Plate Coating and Blocking

Determine the number of wells required. Standards, samples, blanks and/or controls should be analyzed in duplicate.

1. Dilute 1 µl affinity purified antibody (A80-136A) to 100 µl Coating Buffer for each well to be coated. (Example: for 32 wells dilute 34 µl to 3.4 ml)
2. Add 100 µl of diluted antibody to each well.
3. Incubate coated wells at room temperature (20-25°C) for 60 minutes.
4. After incubation, aspirate the antibody solution from each well.
5. Wash FIVE times as described in the Plate Washing section.
6. Add 200 µl of Blocking Solution to each well.
7. Incubate 30 minutes at room temperature (20-25°C).
8. After incubation, remove the Blocking Solution and wash each well FIVE times as described in the Plate Washing section.
9. Proceed with remainder of assay.

Note: Plates can be held for up to 24 hours after the plate coating and blocking steps. Leave Blocking Solution in wells after step 6, cover and place at 2-8°C. To continue assay, remove Blocking Solution, wash and proceed with remainder of assay.

Plate Washing

1. Fill each well with ELISA Wash Solution
2. Remove ELISA Wash Solution by aspiration. Aspirate plate contents.
3. Repeat procedure four additional times for a total of FIVE washes. Blot plate onto paper towels or other absorbent material.

Note: For automated washing, aspirate plate contents from all wells and fill wells with Wash Buffer. Repeat procedure four additional times for a total of FIVE washes. Blot plate onto paper towels or other absorbent material.

Take care to avoid microbial contamination of equipment. Automated plate washers can easily become contaminated thereby causing assay variability.

Standards and Samples

Dilute the standards in Sample/Conjugate Diluent according to the chart below:

Standard	ng/ml	RC80-135-5 (0.5 mg/ml Hemoglobin F)	Sample Diluent
1	400	5 µl	6.25 ml
2	200	500 µl from std 1	500 µl
3	100	500 µl from std 2	500 µl
4	50	500 µl from std 3	500 µl
5	25	500 µl from std 4	500 µl
6	12.5	500 µl from std 5	500 µl
7	6.25	500 µl from std 6	500 µl
8	0	Blank	500 µl

1. Label eight (8) tubes, one for each standard curve point: 400 ng/ml, 200 ng/ml, 100 ng/ml, 50 ng/ml, 25 ng/ml, 12.5 ng/ml, 6.25 ng/ml and 0 ng/ml (blank).
2. Prepare initial dilution of 400 ng/ml by diluting 5 µl of Human Fetal Hemoglobin Calibrator (RC80-135-5) with 6.25 ml of Sample/Conjugate Diluent. Mix well.
3. Pipette 500 µl of Sample/Conjugate Diluent in remaining tubes.
4. Serial dilute the 400 ng/ml standard 1:1 with Sample/Conjugate Diluent. Perform dilution by mixing 500 µl of the previous standard with 500 µl of Sample/Conjugate Diluent. Continue until standard value of 6.25 ng/ml is reached.
5. Use Sample/Conjugate Diluent only as the zero standard value.
6. Dilute the samples, based on the expected concentration of the analyte, to fall within the concentration range of the standards.
7. Transfer 100 µl of standard or sample to assigned wells.
8. Incubate plate 60 minutes at room temperature (20-25°C).
9. After incubation, remove samples and standards and wash FIVE times as described in the Plate Washing section.

HRP Detection Antibody

1. Dilute the HRP Detection Antibody (A80-136P) in Sample/Conjugate Diluent. Recommended starting dilution is 1:100,000.
2. Transfer 100 µl to each well.
3. Incubate 60 minutes at room temperature (20-25°C).
4. After incubation, remove HRP Detection Antibody and wash FIVE times as described in the Plate Washing section.

TMB Substrate Incubation and Reaction Stop

1. Prepare the substrate solution according to the manufacturer's recommendation. TMB substrate in the ELISA Starter Accessory Kit is supplied as a ready to use solution. Only remove the required amount of TMB Substrate Solution for the number of wells being used.
2. Do NOT use a glass pipette to measure the TMB Substrate Solution. Do NOT cover the plate with aluminum foil or metalized mylar. Do NOT return leftover TMB Substrate to bottle. Do NOT contaminate the unused TMB Substrate Solution. If the solution is blue before use, DO NOT USE IT!
3. Add 100 µl of TMB Substrate Solution into each well.
4. Allow the enzymatic color reaction to develop at room temperature (20-25ºC) in the dark for 15 minutes. The substrate reaction yields a blue solution.
5. After 15 minutes, stop the reaction by adding 100 µl of ELISA Stop Solution (0.18 M H_2SO_4). Tap plate gently to mix. The solution in the wells should change from blue to yellow.

Absorbance Measurement

Note: Evaluate the plate within 30 minutes of stopping the reaction.

1. Wipe underside of wells with a lint-free tissue.
2. Measure the absorbance on an ELISA plate reader set at 450 nm.

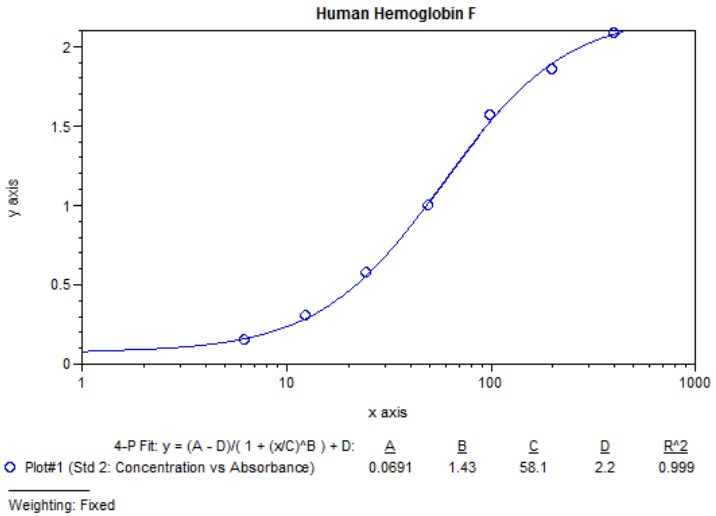
Calculation of Results

1. Duplicate absorbance values should be within 10% of each other. Care should be taken when interpreting data with differences in absorbance values greater than 10%.
2. Prepare a standard curve to determine the amount of Human Fetal Hemoglobin in an unknown sample. Plot the average absorbance value minus the blank value for each standard concentration on the vertical (Y) axis versus the corresponding Human Fetal Hemoglobin concentration on the horizontal (X) axis using graph paper or curve-fitting software.
3. Calculate the Human Fetal Hemoglobin concentration in unknown samples using the prepared standard curve. Determine the amount of Human Fetal Hemoglobin in each unknown sample by noting the Human Fetal Hemoglobin concentration (X axis) that correlates with the absorbance value (Y axis) obtained for the unknown sample.
4. If the sample was diluted, multiply the Human Fetal Hemoglobin concentration obtained by the dilution factor to determine the amount of Human Fetal Hemoglobin in the undiluted sample.

Performance Characteristics

Typical Standard Curve

This typical standard curve was generated using Human Fetal Hemoglobin ELISA Quantitation Set Protocol. This standard curve is for demonstration only. A standard curve must be generated for each assay. Curve was generated as a 4-parameter curve fit using Soft-Max Pro.



Assay Range: 6.25 – 400 ng/ml

Suggested standard curve points are 400 ng/ml, 200 ng/ml, 100 ng/ml, 50 ng/ml, 25 ng/ml, 12.5 ng/ml, 6.25 ng/ml and 0 ng/ml (blank).

Specificity

By immunoelectrophoresis and ELISA the antibodies in this set react specifically with human Fetal Hemoglobin, not with human immunoglobulins or other human serum proteins. Cross-reactivity with other species has not been tested.

Troubleshooting

Problem: Low absorbance
- Incorrect dilutions or pipetting errors
- Improper incubation times
- Wrong filter on microtiter reader. Wavelength should be 450 nm for TMB.
- Set materials or reagents are contaminated or expired.
- Incorrect reagents used.
- Dilute the HRP Detection Antibody less.

Problem: High Absorbance
- Cross contamination from other samples or positive control
- Incorrect dilutions or pipetting errors
- Improper washing
- Wrong filter on microtiter reader. Wavelength should be 450 nm for TMB.
- Contaminated buffers or enzyme substrate
- Improper incubation times
- Set materials or reagents are contaminated or expired.
- Dilute the HRP Detection Antibody more.

Problem: Poor Duplicates
- Poor mixing of specimens
- Incorrect dilutions or pipetting errors
- Technical error
- Inconsistency in following ELISA protocol
- Inefficient washing

Problem: All wells are positive
- Contaminated buffers or enzyme substrate
- Incorrect dilutions or pipetting errors
- Set materials or reagents are contaminated or expired.
- Inefficient washing

Problem: All wells are negative
- Procedure not followed correctly
- Contaminated buffers or enzyme substrate
- Contaminated Conjugate
- Set materials or reagents are contaminated or expired.

Technical Hints

- When preparing coating buffer from the gel capsule, break the capsule apart and pour ingredients into water. Do not place gel capsule into water. The gelatin from the capsule interferes with the binding of the coating antibody to the plate.
- Capture antibody diluted with coating buffer should be added to wells immediately.
- Coated (covered) plates are stable overnight at 4°C.
- Check all buffers for contamination and expiration. When trouble shooting, it may be helpful to start with all new buffers. Make buffers in new or properly cleaned vessels.
- Sodium Azide should not be added to any of the buffers.
- Dilutions should be made shortly before application and immediately applied to appropriate wells. Do not save extra diluted standards or samples for future assays.
- Wash buffer should be aspirated from wells. Pouring/Dumping wash buffer from wells may lead to cross contamination.
- Excess antibody/analyte should be wiped from pipettes tips when making dilutions.
- Incubation time of the TMB Substrate will depend on the intensity of the color change. Stop solution should be added to the plate in the same order as the TMB Substrate.

Warranty

Products are warranted by Bethyl Laboratories, Inc. to meet stated product specifications and to conform to label descriptions when used, handled and stored according to instructions. Unless otherwise stated, this warranty is limited to one year from date of sale. Bethyl Laboratories sole liability for the product is limited to replacement of the product or refund of the purchase price. Bethyl Laboratories products are supplied for research applications. They are not intended for medicinal, diagnostic or therapeutic use. The products may not be resold, modified for resale or used to manufacture commercial products without prior written approval from Bethyl Laboratories, Inc.

Plate Templates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

	1	2	3	4	5	6	7	8	9	10	11	12
A												
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F												
G												
H												

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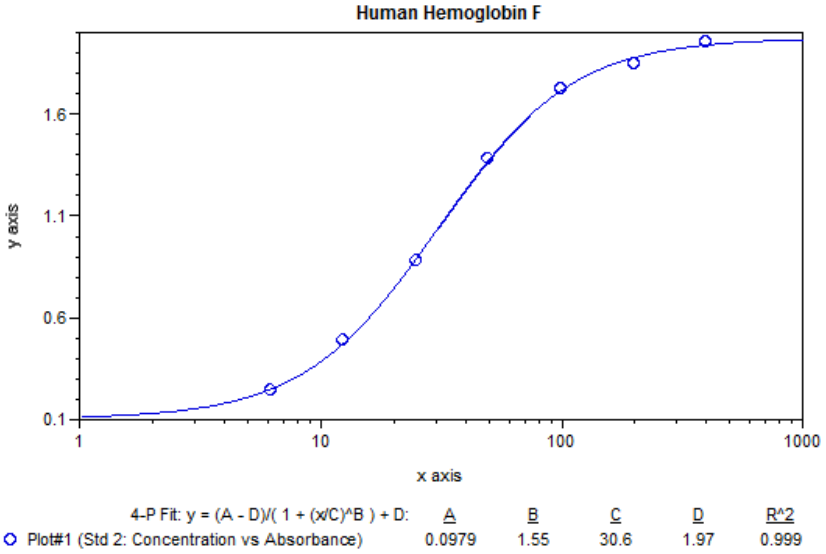

Concentration (ng/ml)	Abs 450 nm	Calculated Concentration
400	1.990	393.23
	1.917	
200	1.884	186.39
	1.809	
100	1.733	104.07
	1.721	
50	1.382	50.49
	1.380	
25	0.881	24.48
	0.867	
12.5	0.499	12.87
	0.473	
6.25	0.250	6.08
	0.220	