



LIFECODES®

LIFECODES® Factor VIII Antibody Screen assay

REF

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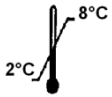


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INTENDED USE

The LIFECODES Factor VIII Antibody Screen is a qualitative solid phase enzyme linked immunosorbent assay (ELISA) designed to detect IgG antibodies reactive with recombinant human factor VIII (FVIII) in human serum and plasma.

SUMMARY AND EXPLANATION

The development of antibodies against human Factor VIII (FVIII) is one of the most detrimental complications in the treatment of hemophilia A, as well as in patients with acquired hemophilia. The antibodies result from a polyclonal response to FVIII and are predominately IgG.¹

The anti-FVIII antibodies can bind to the FVIII in such a way as to block the interactions necessary for FVIII procoagulation activity. These inhibitory antibodies develop in approximately 25% of patients with moderate and severe hemophilia A and can lead to the direct neutralization of any FVIII administered as therapy.¹

In addition, antibodies are also developed to epitopes not associated with FVIII activity. These non-inhibitory antibodies may increase the clearance of FVIII from the circulation, reduce binding to its carrier protein (VWF) or even directly hydrolyze the FVIII molecule.^{2,3,4}

The Factor VIII Antibody Screen utilizes ELISA microwells with immobilized recombinant human FVIII as target molecules for the detection of inhibitory and non-inhibitory antibodies to FVIII.

PRINCIPLE OF THE PROCEDURE

Patient sample is added to microwells coated with recombinant FVIII molecules allowing antibody, if present, to bind. Unbound antibodies are then washed away. An alkaline phosphatase labeled anti-human immunoglobulin reagent (Anti-IgG) is added to the wells and incubated. The unbound Anti-IgG is washed away and the substrate PNPP (p-nitrophenyl phosphate) is added. After a 30-minute incubation period, the reaction is stopped by a sodium hydroxide solution. The optical density of the color that develops is measured in a spectrophotometer.

The Factor VIII Antibody Screen utilizes three different control systems. The negative control is serum collected from a normal, healthy, non-hemophilic donor. The negative control does not contain anti-FVIII antibodies and demonstrates the level of reactivity expected for a negative patient sample. In addition, the negative control is assigned an allowable range of OD values and is used to ensure that proper assay conditions and reagents were used in the assay.

The positive control contains patient serum known to contain FVIII antibodies. This highly reactive sample has a minimum allowable OD value and is used to ensure that proper assay conditions and reagents were used in the assay.

The cutoff between positive and negative samples is set by the reactivity of the kit control. Samples with average OD values greater than the average OD value of the kit control are considered positive. Samples with average OD values equal to or less than the average OD value of the kit control are considered negative.

REAGENTS

Maximum number of tests per kit:

- **F8S: 44 tests per kit**

All reagents should be stored as directed by the label.

	REF	
MS	403571	Microwell Strips: Low-volume flat-bottom microwell strips to which recombinant full-length human FVIII molecules have been immobilized. The microwells are enclosed in a resealable foil pouch. Ready for use.
TCW	403622	Concentrated Wash (10X): Tris (hydroxymethyl) aminomethane buffered solution containing sodium chloride, Tween 20, and 1% sodium azide. Dilute with deionized or distilled water before use. Store Working Wash solution up to 48 hours at room temperature or up to seven days at 2 to 8°C.
SDB	403601	Specimen Diluent: Tris buffered solution containing sodium chloride, bovine serum albumin, and 0.05% sodium azide. Ready for use.
SB	403613	Substrate Buffer: Solution containing diethanolamine, magnesium chloride, and 0.02% sodium azide. Ready for use. Protect from light.

SS	403606	Stopping Solution: 3 M Sodium Hydroxide. Ready for use. Use with care.
AG8	403576	Anti-Human IgG Conjugate: Alkaline phosphatase conjugated goat antibody to human immunoglobulin G (IgG). 0.1% sodium azide. Dilute in Specimen Diluent before use.
PN	403594	PNPP Substrate: (p-nitrophenyl phosphate) Crystalline powder. Reconstitute with deionized or distilled water and dilute in Substrate Buffer before use. Protect from light.
PC1	403596	Positive Control: Human Serum containing bovine serum albumin and 0.1% sodium azide. Dilute in Specimen Diluent before use. CAUTION: Anti-HCV positive (heat inactivated).
KC	403591	Kit Control: Human Serum containing bovine serum albumin and 0.1% sodium azide. Dilute in Specimen Diluent before use. CAUTION: Anti-HCV positive (heat inactivated).
NC1	403593	Negative Control: Human Serum with 0.1% sodium azide. Dilute in Specimen Diluent before use.
PS	503019	Plate Sealers.

PRECAUTIONS

- Do not use reagents that are turbid or appear contaminated.
- Care **MUST** be taken to avoid contamination of Specimen Diluent and Conjugate. Inadvertent contamination of these reagents with human plasma or serum will result in the neutralization of the Conjugate and subsequently to test failure.
- Do not use reagents beyond their expiration date.
- Microwells and reagents contained in the kit are not to be used in conjunction with any other test system.
- Substitution of components other than those provided in this kit may lead to inconsistent or erroneous results.
- Discard any unused portions of diluted Conjugate, diluted Controls, and diluted and reconstituted PNPP reagent after each run.
- When making dilutions, follow pipet manufacturer's instructions for appropriate dispensing and rinsing techniques.
- The enzyme substrate reaction which occurs in the final incubation is temperature and light sensitive and should be performed in a controlled area at 22 to 25°C in the dark.
- Due to variations in instruments or consistently higher or lower room temperatures, it may be necessary for the laboratory to establish a slightly longer or shorter incubation time in order to consistently achieve valid control results. Because the temperature of the final incubation can affect control values, it is important to periodically monitor the room temperature incubation.
- Due to the small size of the low volume microwells, it is essential that the plate reader be checked for proper alignment.
- Do not use a reference wavelength when reading the plate in the plate reader.

CAUTION

- The Positive Control and Kit Control are reactive for Anti-HCV and non-reactive for antibodies to HBsAg and HIV-1. These reagents have been inactivated by heat treatment. However, these materials should still be handled as potentially infectious.
- All human serum used in the Negative Control for this product has been tested and found negative for antibody to HIV, HCV and HBsAg by FDA approved methods. No test method, however, can offer complete assurance that HIV, Hepatitis C virus, Hepatitis B virus or other infectious agents are absent. Therefore, these materials should be handled as potentially infectious.
- Some of the reagents supplied with this kit contain sodium azide as a preservative.
WARNING: Sodium azide reacts with lead and copper plumbing forming highly explosive metal azides. When discarded in a sink, the sink should be flushed with a large volume of water to prevent azide buildup. Sodium azide is a poison and is toxic if ingested.
- Stopping Solution (NaOH) is corrosive. Avoid contact with skin and eyes. Spills should be cleaned up immediately.
- Discard all components when completed according to local regulations.

SPECIMEN COLLECTION

For plasma, the blood should be collected in ACD or sodium citrate using aseptic technique. For serum, the blood should be collected without anticoagulant using aseptic technique. It is recommended that whole blood samples should be collected, transported and processed according to the NCCLS guideline H21-A4, Volume 23, Number 35, December 2003, "Collection, Transport and Processing of Blood Specimens for Coagulation Testing and General Performance of Coagulation Assays." Samples should be tested

while still fresh to minimize the chance of obtaining false positive or false negative reactions due to improper storage or contamination of the specimen. Samples that cannot be tested immediately should be stored at 2 to 8°C for no longer than 48 hours or frozen. Samples frozen at -20°C or below remain in good condition for up to 3 years. However, in order to avoid the deleterious effect of repeated freeze/thaw cycles, it is recommended that samples should be aliquoted in small volumes and then stored frozen. Avoid frost-free freezers.

Particulates or aggregates in the sample can cause false positive results or poor duplicate values. Samples containing particulate matter should be clarified by centrifugation prior to testing.

PROCEDURE

Materials Provided

Vials may contain more reagent than described on the labels. Be sure to measure the reagent with the appropriate device when making dilutions.

1. 12 – 1 x 8 Microwell Strips
2. 1 x 50 mL Concentrated Wash (10X)
3. 1 x 14 mL Specimen Diluent
4. 1 x 14 mL Substrate Buffer
5. 1 x 14 mL Stopping Solution
6. 1 x 30 µL Anti-Human IgG Conjugate
7. 6 x 50 mg PNPP Substrate
8. 1 x 150 µL Positive Control
9. 1 x 150 µL Kit Control
10. 1 x 150 µL Negative Control
11. 12 Plate Sealers

Additional Material Required

1. Test tubes for patient sample, control dilutions and for reagent dilutions
2. Transfer pipets
3. Adjustable micropipets to deliver 1 – 10 µL, 10 – 100 µL and 100 – 1,000 µL and disposable tips
4. Timer
5. Microplate reader capable of measuring OD at 405 or 410 nm
6. Deionized or distilled water
7. Absorbent paper towels
8. Microplate washer or device
9. Centrifuge capable of separating serum or plasma from patient samples
10. 37°C water bath or incubator

Test Procedure

1. Bring all reagents to room temperature.
2. Make Working Wash solution by diluting Concentrated Wash. Add 1 volume of Concentrated Wash to 9 volumes of deionized or distilled water. **Mix well.**
3. Determine the number of patient samples to be tested. Using the Recording Sheet, assign each sample to a location consisting of two (duplicate) wells. Record the identity of each sample on the Recording Sheet.

Prepare Samples and Controls

4. Dilute as follows and mix well:

	Volume Specimen Diluent	Volume Sample
PC1	30 µL	10 µL
KC	30 µL	10 µL
NC1	30 µL	10 µL
Patient Sample	30 µL	10 µL

5. Remove microwell frame from pouch. Promptly remove and reseal unneeded strips in the protective pouch.

NOTE: Only one frame is provided in the kit. Do not discard until all strips have been used.

6. Add 150 µL of Working Wash solution to all wells and allow to stand at room temperature for 5-10 minutes.

7. Aspirate or decant the wash solution and invert on absorbent toweling to prevent drying.
8. Add 15 µL of the appropriate control or sample to the wells as designated on the Recording Sheet.

NOTE: Do not add samples or reagents to blank wells.

NOTE: If multiple patient samples are tested at the same time, only one set of controls is required. **LABEL EACH STRIP TO AVOID ERRORS.**

9. Seal the microwells with a plate sealer and incubate for 30 - 35 minutes in a 37°C water bath. If a dry incubator is used instead, increase time by 10 minutes.
10. Dilute the Conjugate 1 to 100 in Specimen Diluent. Use a polypropylene container.

Strips	2 – 1 x 8	12 – 1 x 8
AG8	4 µL	20 µL
SDB	396 µL	1980 µL

NOTE: Conjugate is viscous. Prime tip 2-3 times in Conjugate before dispensing and rinse after addition to Specimen Diluent. Mix well.

11. WASH STEP

- a) Aspirate or decant contents of each well and blot on absorbent toweling.
- b) Add 150 µL Working Wash solution.
- c) Aspirate or decant.
- d) Repeat steps b + c for a total of 3 or 4 washes.
- e) Vigorously decant to remove all residual wash solution. Invert on absorbent toweling to prevent drying.

NOTE: It is important to completely remove all wash solution after the final wash. However, avoid drying out the plate.

12. Add 15 µL of diluted Conjugate (made in a previous step) to all wells EXCEPT those designated as BLANKS.
13. Seal the microwells with a plate sealer and incubate for 30 - 35 minutes in a 37°C water bath. If a dry incubator is used instead, increase time by 10 minutes.
14. Dissolve PNPP Substrate by adding 0.5 mL deionized or distilled water to the vial. Replace stopper and mix well.
15. Dilute the PNPP 1 in 100 in the Substrate Buffer.

Strips	2 – 1 x 8	12 – 1 x 8
PN	10 µL	60 µL
SB	1 mL	6 mL

Mix well. Protect from light until use.

16. WASH STEP

- a) Aspirate or decant contents of each well and blot on absorbent toweling.
- b) Add 150 µL Working Wash solution.
- c) Aspirate or decant.
- d) Repeat steps b + c for a total of 3 or 4 washes.
- e) Vigorously decant to remove all residual wash solution. Invert on absorbent toweling to prevent drying.

Proceed promptly through next three steps.

17. Add 50 µL of the diluted PNPP solution to all the wells EXCEPT those designated as BLANKS.
18. Allow the microwells to stand in the dark for 30 - 35 minutes at ROOM TEMPERATURE (22 to 25°C).

NOTE: Incubation time and temperature after the addition of PNPP is critical. DO NOT vary the established incubation time or temperature. For consistency, begin timing promptly after addition of the reagent to the first well.

19. Stop the reaction by adding 50 µL of Stopping Solution to each well in the same sequence as the addition of substrate. Add 100 µL of Stopping Solution to the blank wells.

20. Read the absorbance (OD) of each well at 405 or 410 nm. Do not use a reference filter. If the results cannot be read immediately, return the wells to a dark location for up to 30 minutes.
21. Subtract the values obtained in the blank wells from all sample and control wells. Many microplate readers are programmed to automatically perform this step.
22. Record the results on the Recording Sheet.

DETAILS OF CALIBRATION

There is no internationally recognized standard for use in measuring antibodies to Factor VIII. In the Factor VIII Antibody Screen assay, the cutoff between positive and negative samples is set by the reactivity of the kit control. Samples with average OD values greater than the average OD value of the kit control are considered positive. Samples with average OD values equal to or less than the average OD value of the kit control are considered negative. The kit control is lot specific and is extensively tested with the assigned kit reagents to ensure that its use results in the expected reportable results for over 90 test samples.

QUALITY CONTROL

Quality control of the Factor VIII Antibody Screen is built into the test system by the inclusion of Positive and Negative Serum Controls. These controls should be included with each test run to help determine if technical errors or reagent failures have occurred.

Criteria for a valid test:

	Negative Control	Positive Control
Mean OD	$\geq 0.03 - \leq 0.200$	≥ 0.800

OD readings obtained from the duplicate test results of the kit control and the positive samples should fall within 20% of the mean of the two values. Samples whose results are outside of this limit should be re-tested.

NOTE: Poor duplicates can be the result of reagent or sample omission, uneven addition of reagents, uneven temperature during incubations, stray light during the final incubation or cross-well contamination. Failure to test in duplicate may lead to acceptance of erroneous results.

INTERPRETATION OF TEST RESULTS

Test results showing OD values greater than the average of the kit control wells are regarded as positive results. Test results showing OD values less than or equal to the average of the kit control wells are regarded as negative results.

LIMITATIONS

- Erroneous results can occur from bacterial or other contamination of test materials, inadequate incubation periods, inadequate washing or decanting of test wells, exposure of substrate to stray light, omission of test reagents, exposure to higher or lower than prescribed temperature requirements, or omission of steps.
- The results of this assay should not be used as the sole basis for a clinical decision.

SPECIFIC PERFORMANCE CHARACTERISTICS

To ensure suitable reactivity and specificity, each lot of the Factor VIII Antibody Screen is tested prior to release with samples containing FVIII antibodies as well as with samples known to be free of such antibodies.

Precision

The within run, between run, and total imprecision of the Factor VIII Antibody Screen was determined. Eight samples with varying reactivity (negative; low, medium, and high positive) were tested in the Factor VIII Antibody Screen in duplicate in 10 separate assays. To obtain the imprecision of the OD values, the data were analyzed by ANOVA according to the CLSI Document EP-5A2. The calculations are shown in the table below. The results demonstrated $\leq 13\%$ total cv for samples with OD values > 0.600 and $\leq 24\%$ total cv for samples with OD values less than 0.600. In addition, the reportable results were analyzed according to CLSI Document EP12-A. There was 100% agreement between the reportable results within run and between run for each sample tested.

Sample	Mean OD Value	Within Run SD	Within Run %cv	Between Run SD	Between Run %cv	Total SD	Total %cv
Sample 1 Low positive	0.390	0.052	13.3	0.092	23.6	0.099	25.4
Sample 2 Low positive	0.650	0.014	2.2	0.063	9.7	0.064	9.9
Sample 3 Medium positive	0.880	0.014	1.6	0.089	10.1	0.089	10.1
Sample 4 Medium positive	1.120	0.041	3.7	0.086	7.7	0.090	8.0
Sample 5 High positive	1.730	0.094	5.4	0.224	13.0	0.234	13.5
Sample 6 Negative	0.056	0.011	19.7	0.009	16.1	0.012	21.4
Sample 7 Negative	0.057	0.010	17.6	0.011	19.3	0.013	22.8
Sample 8 Negative	0.070	0.011	15.7	0.010	14.3	0.012	17.2

Method Comparison: Comparison of Factor VIII Antibody Screen to Factor VIII Inhibitor Assay

Two separate studies were conducted in which the Factor VIII Antibody Screen was compared to a previously FDA cleared device; GTI Factor VIII Inhibitor Assay. The Factor VIII Antibody Screen is a modified version of the Factor VIII Inhibitor Assay. The results of the two studies were combined. One hundred and thirty seven samples, including serum and plasma, were tested. It was observed during the course of the method comparison study that seven samples from hemophiliac patients with no detectable Bethesda titer and negative reactivity on the Factor VIII Antibody Screen demonstrated unexpected positive reactivity on the Factor VIII Inhibitor Assay. This reactivity was shown to be non-FVIII specific. The Factor VIII Antibody Screen uses a Specimen Diluent which is especially formulated to reduce non-FVIII specific reactivity. When the unexpected positive samples were retested on the Factor VIII Inhibitor Assay in the presence of this reformulated Specimen Diluent, most of the samples showed a significant reduction in overall OD values and a number of the samples demonstrated negative reactivity. The following table shows the results from this study:

Factor VIII Inhibitor Assay

	Positive	Negative	Total
Factor VIII Antibody Screen Positive	89	1	90
Negative	7	40	47
Total	96	41	137

Agreement: 94.2%
 Co-positivity: 92.7% (95% Confidence Interval = 85.7 – 96.4%)
 Co-negativity: 97.6% (95% Confidence Interval = 87.4 – 99.6%)

The Factor VIII Antibody Screen showed excellent sensitivity (co-positivity), specificity (co-negativity), and overall agreement when compared to the Factor VIII Inhibitor Assay.

Method Comparison: Comparison of Factor VIII Antibody Screen to Bethesda Assay

Two separate studies were conducted in which the Factor VIII Antibody Screen was compared to the Bethesda Assay. The results of the two studies were combined. Two hundred and six samples were tested on the Factor VIII Antibody Screen and in the Bethesda Assay or a modified Bethesda Assay (Bethesda Screen). The Bethesda Assay is considered the gold standard for measurement of inhibitory antibodies to Factor VIII. For the purpose of this study, any sample with a positive Bethesda titer was assigned a positive reportable result and any sample with a negative Bethesda titer was assigned a negative reportable result. The following table shows the results from this study:

Bethesda Assay

	Positive	Negative	Total
Factor VIII Antibody Screen Positive	92	12	104
Negative	4	98	102
Total	96	110	206

Agreement: 92.2%
 Co-positivity: 95.8% (95% Confidence Interval = 89.8 – 98.4%)
 Co-negativity: 89.1% (95% Confidence Interval = 81.9 – 93.6%)

The four samples that were Bethesda positive and Factor VIII Antibody Screen negative ranged in Bethesda titer from 0.8 to 3 BU. The Bethesda values were not confirmed in a second laboratory, however the Factor VIII Antibody Screen results were consistently negative for these samples. Twelve samples were shown to be Bethesda negative and Factor VIII Antibody Screen positive. The majority of these samples were from serial tests of the same patient, monitoring either the development of Factor VIII antibodies or the reduction in Factor VIII antibodies during immune tolerance therapy. In general, these discrepancies were found in samples taken from the patient immediately prior to a patient becoming Bethesda positive or immediately following a patient becoming Bethesda

negative, suggesting an increased sensitivity to Factor VIII antibodies in the LIFECODES Factor VIII Antibody Screen. The Factor VIII Antibody Screen showed excellent sensitivity (co-positivity), specificity (co-negativity), and overall agreement when compared to the gold standard, Bethesda Assay.

Interfering Substances

The following substances showed no interference in the Factor VIII Antibody Screen at the concentrations indicated:

Hemoglobin	< 500 mg/dL
Bilirubin	< 20 mg/dL
Intralipid	< 500 mg/dL
Gammagard (IVIG)	< 200 µg/dL
Rituxan (rituximab)	< 10 µg/dL

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