PrecisionBioLogic

CRYO*check*™ IVD

Factor VIII Inhibitor Kit

Intended Use

The CRYO*check* FVIII Inhibitor Kit is for clinical laboratory use in conjunction with a factor VIII activity assay to enable performance of a modified Nijmegen-Bethesda assay using 3.2% citrated human plasma. It enables the determination of a functional FVIII inhibitor titer to aid in the clinical management of congenital hemophilia A in individuals aged 2 years and older. For in vitro diagnostic use.

Summary and Principle

The FVIII Inhibitor Kit is used to prepare samples for the Centers for Disease Control and Prevention (CDC) modification of the Nijmegen-Bethesda assay[1]. Factor VIII (FVIII) inhibitors are antibodies that neutralize FVIII activity in a time and temperature dependent manner. In the CDC modification of the Nijmegen-Bethesda assay, a heat inactivation of the test plasma is performed to inactivate endogenous FVIII, leaving any FVIII inhibitor antibodies intact. The inactivated test plasma is then incubated with an exogenous FVIII source (Imidazole Buffered Pooled Normal Plasma), during which time FVIII inhibitor, if present, will progressively neutralize the FVIII. By standardizing the amount of FVIII added and the incubation time, the activity of the inhibitor in the test plasma can subsequently be determined according to how much of the added FVIII is inactivated relative to a control mixture as measured by a FVIII activity assay[2].

FVIII inhibitor testing is used to identify and monitor the presence of FVIII inhibitory antibodies that can occur because of the use of FVIII replacement therapies for treatment of persons with hemophilia A. The presence of inhibitors results in a loss of effectiveness of a particular therapy; thus, detecting and monitoring FVIII inhibitor activity is important in the clinical management of persons with hemophilia A.

Reagents

- **Imidazole Buffered Pooled Normal Plasma (IB-PNP):** Pooled normal plasma from a minimum of 20 donors with a FVIII activity value of 95-113% and buffered with imidazole to a pH of 7.3-7.5.
- **Imidazole Buffered Bovine Serum Albumin (IB-BSA):** A 4% BSA solution buffered with imidazole to a pH of 7.3-7.5.
- **Negative Factor VIII Inhibitor Control:** Pooled normal plasma from a minimum of five donors buffered with HEPES to a pH of 6.2-8.2.
- **Positive Factor VIII Inhibitor Control:** HEPES buffered (pH 6.2-8.2) immunodepleted FVIII deficient plasma to which anti-human FVIII antibodies have been added.

FOR PRESCRIPTION USE ONLY

Storage, Preparation and Handling

When stored at -70 °C, CRYO*check* Factor VIII Inhibitor Kit is stable to the end of the month indicated on the product packaging.

Thaw one vial set per patient sample (see *Availability* table for vial set description) at 37 °C (± 1 °C) in a waterbath using the waterbath "floatie" thawing device (available separately). Thawing times are important and should be strictly adhered to. **The use of a dry bath or heating block for thawing is not recommended.** The use of a timer is recommended. Refer to the Thawing Table for recommended thawing times according to aliquot size. Invert each vial gently prior to use.

Thawing Table	
Aliquot Size	**37 °C (± 1 °C) Waterbath**
1.5 mL	5 minutes
0.5 mL	3 minutes

CRYO*check* FVIII Inhibitor Kit may be used for up to four hours after preparation. When not in use, CRYO*check* FVIII Inhibitor Kit should be capped in the original vials and maintained at 2 to 8 °C. Allow refrigerated components to acclimate to room temperature (18 to 25 °C) and invert gently prior to use.

NB: CRYO*check Factor VIII Inhibitor Kit components are lot-specific and should not be interchanged with other lot numbers.*

Availability

Product	Catalog #	Format 5 Vial Sets – Each Set Containing
CRYO*check* FVIII Inhibitor Kit	CCIK08	IB-PNP 2 x 1.5 mL (blue cap) IB-BSA 2 x 1.5 mL (orange cap) Negative FVIII Inhibitor Control 1 x 0.5 mL (green cap) Positive FVIII Inhibitor Control 1 x 0.5 mL (red cap)

Instruments

Each lab should prepare the local instrument in accordance with the manufacturer's instructions for use.

Procedure

Materials Provided

- CRYO*check* FVIII Inhibitor Kit

Materials Required but not Provided

- Waterbath capable of maintaining 56 °C (± 1 °C)
- Waterbath capable of maintaining 37 °C (± 1 °C)
- Floatie for thawing vials in waterbath
- Microcentrifuge
- Ice bath
- Coagulation instrument or assay system
- FVIII activity assay and associated calibration and control plasma as applicable
- Plastic test tubes with caps (e.g. 12 x 75 mm)

- Coagulation reaction cuvettes
- Micropipette capable of pipetting 200 µl
- Timer

Specimen Collection and Preparation

Patient samples should be collected into 105 – 109 mmol/L sodium citrate dihydrate anticoagulant (3.2%) in a ratio of 9 parts blood to 1 part anticoagulant in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines[3]. Patient plasma is derived by centrifugation at 1500 x g for 15 minutes in order to achieve platelet-poor plasma (< 10,000 platelets/µL) and should be tested within four hours of collection when maintained at 2 to 4 °C. If samples are not to be tested within four hours, then plasma should be removed from the cells and frozen at -20 °C for up to two weeks or -70 °C for up to twelve months. Samples should not undergo more than two freeze-thaw cycles prior to testing.

Assay Procedure

1. Prepare CRYO*check* Factor VIII Inhibitor Kit reagents according to Storage, Preparation and Handling instructions above. Prepare one vial set per patient sample (see Availability). Take extra care to ensure that vial types are kept separate to avoid potential sample preparation error.
2. Prepare instrument according to the manufacturer's instructions for use.
3. Prepare FVIII activity assay according to the manufacturer's instructions for use.
4. Heat inactivation of test samples (patient sample, Positive FVIII Inhibitor Control and Negative FVIII Inhibitor Control):
    - Incubate test samples at 56 °C for 30 minutes in a waterbath to inactivate FVIII present in the plasma sample before testing.
    - Centrifuge test samples at room temperature at 2700 x g for five minutes and transfer supernatant to appropriately labeled vials.
5. Prepare dilutions of heat inactivated test samples (patient sample, Positive FVIII Inhibitor Control, and Negative FVIII Inhibitor Control). *See Test Sample Dilution Table below*:
    - For patient sample, if a FVIII inhibitor is expected, test samples are diluted with IB-BSA to achieve a sample that is close to 50% residual FVIII activity (see Results and Interpretation, below, for definition).
    - The Positive FVIII Inhibitor Control is diluted with IB-BSA in order to achieve approximately 50% residual FVIII activity.
    - The Negative FVIII Inhibitor Control does not require dilution and should be tested neat.

Test Sample Dilution Table

Sample / Control	Vial	Test Vial	IB-BSA	Test Sample / Sample Dilution	IB-PNP
Control Mix	1	Control mix	0.20 mL		0.20 mL
Negative FVIII Inhibitor Control	1	Neat		0.20 mL of Negative Control	0.20 mL
Positive FVIII Inhibitor Control	1	Neat		0.20 mL of Positive Control	0.20 mL
	2*	½	0.20 mL	0.20 mL of Positive Control	0.20 mL
Test Sample	1	Neat		0.20 mL of sample	0.20 mL
	2	½	0.20 mL	0.20 mL of sample	0.20 mL
	3	¼	0.20 mL	0.20 mL of vial 2	0.20 mL
	8*	1/128	0.20 mL	0.20 mL of vial 7	0.20 mL

*Discard 0.20 mL after mixing IB-BSA with test sample and before adding IB-PNP

*Note: This is an **example only** of a serial dilution profile. Dilutions should be prepared until a residual activity of 50% is reached.*

6. Prepare test mixes (patient sample, Positive FVIII Inhibitor Control, and Negative FVIII Inhibitor Control):
   - Prepare a 1:1 mix of each undiluted (neat) test sample and IB-PNP (test mix).
   - Prepare a 1:1 mix of each dilution of each test sample (if applicable) with IB-PNP. The addition of IB-PNP provides a consistent amount of FVIII to each test mix.
   - Invert vial gently five times to mix.
7. Prepare a control mix:
   - Prepare a 1:1 control mix of IB-BSA and IB-PNP.
   - Invert vial gently five times to mix.
8. Incubate all test mixes prepared in step six and control mix prepared in step seven at 37 °C for two hours in a waterbath. If a FVIII inhibitor antibody is present, it will neutralize the FVIII that was added to the test mix as IB-PNP.
9. After the two-hour incubation, place the test mixes and the control mix in an ice bath for 10 minutes to halt the reaction or perform a FVIII assay immediately.
10. Measure FVIII activity in all test mixes and the control mix using a FVIII activity assay on a coagulation analyzer.

Results and Interpretation

Residual FVIII activity is converted to Bethesda Units (BU)/mL, with one BU defined as the amount of inhibitor that results in 50% residual FVIII activity, using the formula: BU = [(2 – Log %RA) / 0.30103] x dilution factor.

Calculate residual FVIII activity (RA) using the following formula:

%RA = (test mix % FVIII activity / control mix % FVIII activity) *100

Convert residual FVIII activity to Bethesda units (BU)/mL using the formula:

BU = [(2 – Log %RA) / 0.30103] x dilution factor

For example, 100% RA = 0 BU/mL and 50% RA =1 BU/mL.

Interpretation

- Results should be expressed in BU/mL
- Samples ≥ 0.6 BU/mL are considered positive for FVIII inhibitor[4].

Quality Control

Each laboratory should establish its own quality control (QC) ranges for FVIII activity assay using acceptable statistical methods. These QC ranges may then be used to monitor and validate the integrity of the testing system[5]. For all coagulation tests, the laboratory must include at least two levels of control for every eight hours of operation and any time a change in reagents occurs[6].

Quality control of the Factor VIII Inhibitor Kit is built into the test system by the inclusion of the Positive Factor VIII Inhibitor Control and the Negative FVIII Inhibitor Control. These controls should be included in each test run to help identify if errors or reagent failures have occurred.

Expected Values

Expected results for the FVIII Inhibitor Kit controls are as follows:

- Negative Factor VIII Inhibitor Control: < 0.6 BU/mL
- Positive Factor VIII Inhibitor Control: ≥ 0.6 BU/mL.

Refer to the Assay Certificate for the expected ranges specific to each lot number of CRYOcheck Factor VIII Inhibitor Kit.

Performance Characteristics

All studies were performed using the CRYO*check* FVIII Inhibitor Kit in conjunction with the Siemens FVIII chromogenic assay on Siemens BCS XP instruments.

Method Comparison

A method comparison study was conducted at three sites (one internal and two external) according to CLSI EP09c to compare the accuracy of the CRYO*check* FVIII Inhibitor Kit relative to a comparator device[7]. Aliquots of human plasma samples from individuals with congenital hemophilia A (N=210) were distributed across three sites and tested using a single lot of CRYO*check* FVIII Inhibitor Kit. A second aliquot of each sample was tested at a central reference laboratory using a chromogenic CDC-Modified Nijmegen-Bethesda Assay that has been implemented as a validated Laboratory-Developed Test on a Siemens BCS XP instrument. The data demonstrated positive agreement of 100% (95% CI, 97-100%) and negative agreement of 99% (95% CI, 93-100%), as summarized below.

		CRYO*check* FVIII Inhibitor Kit results		
		Positive	**Negative**	**Total**
Comparator device results	**Positive**	133	0	133
	Negative	1	76	77
	Total	134	76	210

Results were compared by Passing-Bablok regression analysis and Bland-Altman plots. Regression statistics show that the CRYO*check* FVIII Inhibitor Kit performed equivalently to the comparator method.

	N	Slope		Intercept		Pearson Correlation Coefficient
		Value	95% CI	Value	95% CI	
Site 1	68	1.543	1.404, 1.660	-0.224	-0.434, -0.127	0.977 (r^2=0.954)
Site 2	72	1.065	0.998, 1.190	-0.113	-0.138, -0.043	0.985 (r^2=0.969)
Site 3	70	1.373	1.225, 1.455	-0.075	-0.159, -0.023	0.980 (r^2=0.960)
Overall	210	1.341	1.265, 1.406	-0.145	-0.180, -0.070	0.970 (r^2=0.940)

Absolute predicted biases at medical decision levels are reported below

Titer (BU/mL)	Predicted Bias (BU/mL)	Lower CI (%)	Upper CI (%)
0.6	-2.0	-4.8	0.8
1	-1.9	-4.6	0.9
5	-0.3	-3.0	2.5
10	1.7	-0.9	4.4

Limit of Blank, Limit of Detection and Limit of Quantification

The limit of blank (LoB) was determined following the CLSI EP17-A2 guideline by measuring four blank plasma samples obtained from normal healthy donors[8]. Samples were measured in triplicate using three lots of CRYO*check* FVIII Inhibitor Kit over five days. The LoB was determined to be 0.1 BU/mL.

The limit of detection (LoD) was determined following the CLSI EP17-A2 guideline by measuring four low titer plasma samples obtained from congenital hemophilia A donors[8]. Samples were measured in triplicate using three lots of CRYO*check* FVIII Inhibitor Kit over five days. The LoD was determined to be 0.2 BU/mL.

The limit of quantitation (LoQ) study was determined according to the CLSI EP17- A2 guideline[8]. Aliquots of each low titer sample used in the LoD study were sent to an external laboratory for testing in three replicates on three different days to determine assigned values using a validated, laboratory-developed, CDC-modified Nijmegen- Bethesda assay. The LoQ was determined to be 0.2 BU/mL.

Linearity

A linearity study was conducted in accordance with CLSI EP06-A using one lot of CRYO*check* FVIII Inhibitor Kit[9]. Factor VIII inhibitor positive plasma from a congenital hemophilia A patient was combined with congenital hemophilia A patient plasma containing no FVIII inhibitor to create twelve sample dilutions with estimated titers in the range of 0 to 100 BU/mL. An expanded linearity study was also conducted, in which a series of eighteen sample dilutions comprised of FVIII monoclonal antibody in immunodepleted FVIII deficient plasma with titers in the range of 0 to 1402.9 BU/mL were tested. The results of these two studies support a linearity range of 0.2 to 1402.9 BU/mL.

Precision

An internal precision study was performed using three different lots of CRYO*check* FVIII Inhibitor Kit by one operator on a Siemens BCS XP analyzer in accordance with CLSI EP05-A3[10]. The study quantified the kit's Positive Factor VIII Inhibitor Control and Negative Factor VIII Inhibitor Control as well as four plasma samples from congenital hemophilia A patients representing negative, low, mid, and high levels of FVIII inhibitor. Each sample was measured with each product lot in duplicate, twice a day for 20 days for a total of 80 replicates per sample per lot. The results demonstrated a pooled precision of 8% CV for the positive samples and 0.1 BU/mL SD for the negative sample.

Sample	Mean Value (BU/mL)	Within-Laboratory Precision	
		SD	%CV
Negative Plasma Sample	0.3	0.1	N/A
Low Plasma Sample	1.2	0.1	8.2
Mid Plasma Sample	5.3	0.4	8.3
High Plasma Sample	8.6	0.7	8.4

Reproducibility

Reproducibility studies were conducted at three sites (one internal and two external) by three different operators on three different Siemens BCS XP analyzers using a single lot of CRYO*check* FVIII Inhibitor Kit in accordance with CLSI EP05-A3. The study quantified the kit's Positive Factor VIII Inhibitor Control and Negative Factor VIII Inhibitor Control as well as four plasma samples from congenital hemophilia A patients representing negative, low, mid, and high levels of FVIII inhibitor. Each sample was measured in triplicate, twice a day for five days for a total of 30 replicates per sample per site. The data across three sites demonstrated a pooled reproducibility of $\leq$ 16% CV for the positive samples and 0.1 BU/mL SD for the negative plasma sample as summarized below.

Sample	Mean Value (BU/mL)	Within-Run (Repeatability)		Between-Run		Between-Day		Between-Site		Reproducibility	
		SD	%CV	SD	%CV	SD	%CV	SD	%CV	SD	%CV
Negative FVIII Inhibitor Control	0.0	0.1	N/A	0.0	N/A	0.0	N/A	0.0	N/A	0.1	N/A
Positive FVIII Inhibitor Control	1.8	0.2	8.9	0.1	3.3	0.1	3.4	0.2	12.4	0.3	16.0
Negative Plasma Sample	0.3	0.1	N/A	0.0	N/A	0.0	N/A	0.0	N/A	0.1	N/A
Low Plasma Sample	1.4	0.1	10.2	0.0	0.0	0.0	2.9	0.0	0.0	0.1	10.6
Mid Plasma Sample	5.5	0.4	7.8	0.1	1.9	0.1	2.2	0.0	0.0	0.5	8.3
High Plasma Sample	9.6	0.7	7.8	0.4	4.0	0.3	3.4	0.9	9.2	1.3	13.2

Interferences

Interference studies were conducted according to CLSI EP7-A2 using a single lot of CRYO*check* FVIII Inhibitor Kit[11]. Patient plasma samples were spiked with possible interferents, and 10 replicates were tested alongside 10 replicates of the corresponding blank matrix control. The following substances showed no interference up to the concentrations indicated:

Substance Tested	Test Concentration
Hemoglobin	≤ 500mg/dL
Bilirubin	≤ 29mg/dL
Intralipid	≤ 500mg/dL
von Willebrand factor	≤ 20 µg/mL

Lupus anticoagulant autoantibodies may interfere with the quantification of low titer FVIII inhibitors.

Rheumatoid Factor at ≥ 82 IU/mL showed interference with the quantification of FVIII inhibitors.

Precautions/ Warnings

Do not use the product if it is thawed upon receipt or if the vials appear cracked. Transferring the material into another container other than siliconized glass or polypropylene could have a performance impact and is not recommended.

Any serious incident that has occurred in relation to the use of this device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or patient is established.

All blood products should be treated as potentially infectious. Source material from which this product was derived was found to be negative when tested in accordance with current required tests for transfusion-transmitted diseases. No known test methods can offer assurance that products derived from human blood will not transmit infectious agents. Accordingly, these human blood-based products should be handled and discarded as recommended for any potentially infectious human specimen[12].

Bibliography

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Symbols Used

IVD	In vitro diagnostic medical device		Biological risks
LOT	Batch code		Manufacturer
REF	Catalogue number	EC REP	Authorized representative in the European Community / European Union
	Use by date	**Rx ONLY**	For prescription use only
	Upper temperature limit		Consult electronic instructions for use

EC REP European Authorized Representative (Regulatory affairs only)
Emergo Europe— Westervoortsedijk 60, 6827 AT Arnhem, The Netherlands

CE

UKRP United Kingdom Responsible Person
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