

BIOPHEN DiXal (Direct Xa Inhibitors) Ref 221030

Chromogenic assay for Direct Factor Xa inhibitors (DiXals)

For in vitro diagnostic use only

ANIARA

Manufactured By: HYPHEN BioMed

Last revision: 10/10/2011

INTENDED USE:

BIOPHEN DiXal kit is a chromogenic assay for in vitro quantitative measurement of Factor Xa Direct Inhibitors (DiXals), such as Rivaroxaban, on human citrated plasma (or purified milieu), using a manual or automated method.

This method is not appropriate for indirect inhibitors such as Fondaparinux or heparins.

CLINICAL APPLICATIONS:

Laboratory monitoring of Direct Factor Xa (FXa) inhibitors, and especially Rivaroxaban; when it is required. Measuring the DiXal concentration in patients' plasma allows monitoring the therapy and adjusting drug dosage. This assay is insensitive to heparins.

ASSAY PRINCIPLE:

Biophen DiXal is a two stage method based on the inhibition of a constant and in excess amount of exogenous Factor Xa (FXa), by the tested DiXal, and hydrolysis of a Factor Xa specific chromogenic substrate, by the residual factor Xa. pNA is then released from the substrate. The amount of pNA released is a direct relationship of the residual factor Xa activity. There is an inverse relationship between the concentration of DiXal in the tested sample and color development, measured at 405 nm.

[DiXal] + [FXa (excess)] → [FXa-DiXal] + [residual FXa]
[FXa (residual)] + Substrate → Peptide + pNA

SPECIMEN:

Human plasma prepared from citrated anticoagulated blood, where FXa direct inhibitors (eg Rivaroxaban) must be measured. Alternatively, DiXal can be assayed in purified milieu.

REAGENTS:

Biophen DiXal kit contains 3 vials of FXa, 3 vials of a specific Factor Xa substrate, and 4 vials of buffer.

R1: Reagent 1: FXa (h)

Purified human FXa, lyophilised in presence of stabilizers.
3 vials (each vial to be reconstituted with 2.5 mL of distilled water).

R2: Reagent 2: Substrate

FXa specific chromogenic substrate (CS-11(65)), lyophilised in presence of mannitol.
3 vials (each vial to be reconstituted with 2.5 mL of distilled water).

R3: Reagent 3: Buffer

Tris-NaCl-EDTA assay reaction buffer at pH 7.85, containing 1% PEG6000 and sodium azide as preservative. Ready to use. 4 vials of 25 mL.

Warning:

- FXa was prepared from human plasma, which was tested with registered methods and found negative for HIV antibodies, HBs Ag and HVC antibodies. Bovine Serum Albumin (BSA) was prepared from bovine plasma, which was tested for the absence of infectious agents, and collected from animals free from BSE. However, no assay may warrant the total absence of infectious agents. Any product of biological origin must then be handled with all the required cautions, as being potentially infectious.
- Sodium azide (0.9 g/l) may react with lead and copper plumbing to form highly explosive metal azides. Flush with large volumes of water when discarding into a sink.
- FXa concentration is adjusted if required for each lot for providing the right reactivity and linearity in the assay

REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED:

Reagents:

- Distilled water, preferentially sterile.
- Acetic Acid (20%) or Citric Acid (2%) (End point method).
- Calibration plasmas titrated for the assayed DiXal
- Or assayed DiXal Reference Material (international or internal)
- and citrated normal human plasma pool collected with great care, in order to avoid activation, to prepare the calibration curve.
- Suitable Quality Controls titrated for the assayed DiXal.
- Biophen Rivaroxaban Control (ref : 224501)
- Biophen Rivaroxaban plasma calibrator (ref : 222701)

Material:

- Spectrophotometer, photometer or automates for chromogenic assays, with a wave-length set up at 405 nm.
- Stop watch.
- Calibrated pipettes.

TRACEABILITY TO THE REFERENCE MATERIAL:

The kit reactivity is standardized by reference to a DiXal preparation, such as Rivaroxaban.

STORAGE CONDITIONS:

Reagents must be stored at 2-8°C, in their original packaging box. They are then stable until the expiration date printed on the box.

Note: Stability studies for 3 weeks at 30°C show that the reagents can be shipped at room temperature for a short period without damage.

PREPARATION AND STABILITY OF REAGENTS:

Note: Reconstitution volumes can vary according to the automate used. Refer to each specific instrument adaptation.

R1: Reagent 1: Human FXa

Reconstitute each vial with exactly 2.5mL of distilled water. Shake thoroughly until complete dissolution of the content (vortex). Let to homogenize for 30 minutes at room temperature (18-25 °C), while shaking the vial from time to time.

Homogenize the content before each use.

Stability of restored reagent R1, provided that any contamination or evaporation is avoided, kept in its original vial or in a plastic tube:

- 15 days at 2-8°C.
- 7 days at room temperature (18-25 °C).
- 2 months at -20°C or below (before use thaw in a water bath at 37°C for at least 15min).

R2: Reagent 2: FXa specific chromogenic substrate

Reconstitute each vial with exactly 2.5 mL of distilled water. Shake thoroughly until complete dissolution of the content (vortex). Incubate at room temperature (18-25°C) for 30 minutes, while shaking the vial from time to time (vortex). Check that the substrate is totally dissolved before use.

Homogenize the content before each use.

Stability of restored R2, provided that any contamination or evaporation is avoided, kept in its original vial or in a plastic tube:

- 2 months at 2-8°C.
- 7 days at room temperature (18-25 °C).
- 2 months at -20°C or below (before use thaw in a water bath at 37°C for at least 15 min).

R3: Reagent 3: Buffer

Ready to use buffer (25 ml vials). Shake before use.

Stable until the expiration date, stored at 2-8°C, protected from any contamination.

Stability of the buffer when open, in its original vial:

- 2 months at 2-8°C.
- 7 days at room temperature (18-25°C).

Cautions:

- In order to improve stability, reagents must be closed with their original screw cap following each use (white cap for R1, yellow cap for R2, and white cap for buffer R3).
- Reagents must be handled with care, in order to avoid any contamination during use.
- If the substrate becomes yellow, this indicates the presence of a contaminant. It must be rejected, and a new vial must be used.
- Incubating the reconstituted vials at RT allows stabilizing the reagents, and obtaining a homogeneous reactivity.
- Take care to limit as much as possible any evaporation of the reagents during use, eg. by using chimneys.

Note:

- R1, R2 vials are closed under vacuum. Remove carefully the stopper, in order to avoid any lost of powder when opening the vials.
- According to the automated method used, the reagents can be reconstituted with volumes different from those recommended. In any case, the established reactive ratios (respective reagent concentrations in the reactive milieu) between reagents must be adhered to.
- Use only reagents from kits with the same lot number. Do not mix reagents from kits with different lots when running the assay. Reagents are optimized for each lot of kits.

SPECIMEN COLLECTION:

Blood (9 vol.) must be collected on 0.109M (or 0.129M) trisodium citrate anticoagulant (1 vol.) through a net venipuncture, and the first drops must be discarded.

- Within 4 hours, blood must be centrifuged at 2,500 g for 15 min at room temperature (18-22°C), and plasma decanted into a plastic tube, using a plastic pipette.

Storage of plasma:

- Up to 4 hours at room temperature (18-25°C)
- Up to 1 month frozen at -20°C or below (before use, thaw for 15 min. in a water bath at 37°C).

Note: Refer to GEHT or NCCLS/CLSI recommendations for further instructions on specimen collection, handling and storage. Discard any plasma presenting an unusual aspect (haemolysed, lipaemic aspect...).

TEST PROCEDURE:

Biophen DiXal kit is designed for being used with automated kinetics methods but it can also be used for end point manual methods. Adaptations to the various automates are available upon request. The assay is performed at the controlled temperature of 37°C and the colour development is measured at 405 nm.

D750-02/BI/1030

ANIARA

8580 Gove Court • Mason, OH 45040

Phone: 513.770.1991

Toll Free: 866.783.3797

Fax: 513.573.9241

Email: info@aniara.com

www.aniara.com

CALIBRATION:

Using the Biophen Rivaroxaban Calibrator (222701) or the Rivaroxaban (DiXal) reference material (eg from a Rivaroxaban stock solution at 1mM or 436µg/ml in DMSO, prepare an intermediate stock solution at 25 µg/ml (dilution 1: 17.44) in R3 buffer supplemented with 5%DMSO and 0.2%BSA), prepare a calibration curve in normal citrated human plasma pool (for assayed plasma samples) or in R3 buffer (for assay in purified milieu), as follows:

Dilute the stock solution at 25 µg/ml at 1:50 in plasma for getting the calibrator at 0.5 µg/ml. Dilute this calibrator 1:2 with plasma for getting the calibrator at 0.25 µg/ml.

Rivaroxaban (µg/ml):	0	0.25	0.50
----------------------	---	------	------

In order to get the full assay performances, the calibration curve must be prepared just before running the assay.

If other DiXal activities are used, the assay range must be adjusted.

TESTED SAMPLES AND CONTROLS:

Samples and controls are assayed at the 1:20 dilution in R3 Buffer. The diluted samples must be tested within 1 hour.

ASSAY PROTOCOL:

Manual Method:

The assay uses a 1:20 dilution in R3 buffer for the assayed plasma or controls, or for calibrators.

Into the microwell or the plastic test tube, incubated at 37°C, introduce:

Reagents	Microplate	Test Tube
Calibrators, or diluted tested plasmas or Controls (1:20 in R3)	50 µl	200 µl
R1 : Human FXa preincubated at 37°C	50 µl	200 µl
Mix and incubate for 1 min at 37°C, then introduce:		
R2: Substrate preincubated at 37°C	50 µl	200 µl
Mix and incubate for 45 seconds at 37°C, exactly		
Stop the reaction by introducing:		
Citric Acid (20 g/L) or 20 % Acetic Acid	50 µl	200 µl
Mix and measure the Absorbance at 405nm against the sample blank.		

The yellow colour obtained is stable for 1 hour.

The sample blank is obtained by mixing the reagents in the reverse order from that of the test i.e.: Citric Acid (20 g/L), substrate, FXa, diluted sample.

Measure the Absorbance at 405 nm (A405). Subtract the sample blank from the A405 obtained for the assay.

Kinetics mode:

The assay can be read using a kinetics mode. In this case the change in absorbance is recorded from 10 to 35 seconds following the addition of substrate. There is then no need to subtract the sample blank, or to stop the reaction. The results are obtained using the change in absorbance ($\Delta A405$) for calibrators and tested specimen.

Automated methods:

Adaptations to the various analysers are available upon request. The assay is then performed kinetically. The reaction does not require to be stopped and sample blanks are automatically subtracted. Reconstitution volumes can vary according to the automate used. Refer to each specific adaptation and specific cautions for each instrument.

Note:

If higher or lower reactive volumes than those indicated here above are required for the method used, the same respective proportions between reagent concentrations and volumes used, must be adhered to, in order to maintain the assay performances.

Run a sample blank in presence of highly lipemic, icteric or haemolysed plasmas, or if the plasmas has a "colour" different from the usual one.

QUALITY CONTROL:

Using appropriate quality controls such as Biophen Rivaroxaban Control (224501), with a known concentration of the assayed DiXal, allows validating the calibration curve, as well as the homogeneous reactivity from run to run, when using a same lot of reagents. The calibration curve is acceptable when the concentrations measured for controls are within the acceptance range. Each laboratory should verify its own target value and acceptance range, in the exact working conditions, for each new lot of controls.

Note: A new calibration curve must be carried out for each new lot of reagents, after each important maintenance of the analyzer, or when measured values for the quality controls are out of the acceptance range determined for the method. Each laboratory can establish its own acceptance ranges, according to the instruments and protocols used. Include at least one quality control (at different levels) in each test series.

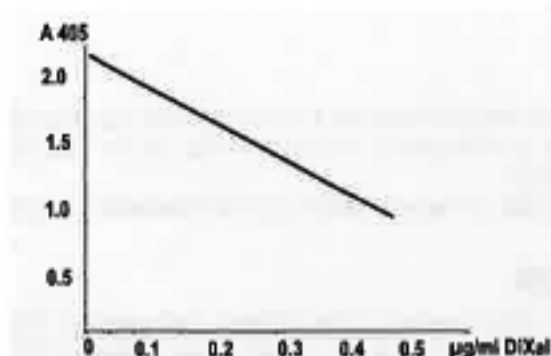
RESULTS:

- For the end-point method, use a linear graph paper and plot on abscissae the DiXal concentration (eg. in µg/ml) and on ordinates the corresponding absorbances (A405). Alternatively, statistics software can be used for establishing the dose response calibration curve.
- An inverse linear relationship is obtained between the DiXal concentrations and Absorbances (A405).
- Draw the calibration curve obtained. Calculate the r^2 value. Calibration is acceptable if: $r^2 > 0.98$, and if measured values for controls are in compliance.
- The DiXal concentration in the tested sample (when assayed diluted 1:20) is directly obtained on the calibration curve. Results are expressed eg. as µg/ml of DiXal.
- When the kinetics mode is used, proceed the same way by plotting the $\Delta A405$ values obtained, instead of A405.
- Using automated methods, the concentrations are directly calculated by the analyser, respectively to the calibration curve, and the sample dilution used.
- The dynamic range is from 0 to 0.5µg/ml of Rivaroxaban.

When the assay dilution is 1:20, the DiXal concentration is directly read on the calibration curve. When other predilutions are used, multiply the measured concentration by the complementary pre-dilution factor in order to get the concentration in the tested specimen.

EXAMPLE OF CALIBRATION CURVE:

The calibration curve below is an example only, obtained for Rivaroxaban in plasma, using the manual (water bath) method. Only the calibration curve generated for the series of assays performed must be used for calculating the concentrations.



PERFORMANCE CHARACTERISTICS:

- The dynamic range is from 0 to about 0.5 µg/ml for Rivaroxaban in plasma.
- The detection threshold for the assay is evaluated on the calibration curve by measuring the "apparent" DiXal concentration, which corresponds to the mean A405 value obtained for a sample free of DiXal less 3 Standard Deviations (SD). This detection threshold is of about 0.05 µg/ml for Rivaroxaban.
- Example of reproducibility results:
 - Intra assay CV < 3%
 - Inter assay CV < 10-15%.
- This assay was designed for avoiding the interference of plasma factors. The assay is totally insensitive to the presence of heparin-like indirect anti-FXa activities such as UFH, LMWH, Fondaparinux/Arixtra®, Sodium Danaparoid at usual therapeutic doses.
- This assay is specific, sensitive, and offers high flexibility for the dynamic range by adjusting the working dilution used.
- For an optimal therapeutic efficacy, the measured DiXal concentration must be within the therapeutic range defined by the manufacturer for the corresponding indication.
- The assay is calibrated and optimised for Rivaroxaban. The curves are constructed respectively to the Rivaroxaban concentration, expressed in µg/mL. If a calibration in anti-Xa units/mL is needed, or when a different FXa direct inhibitor is used, the user must take into account the specific anti-Xa activity of the substance used.
- Limitations of the procedure:
 - Blood activation, during specimen collection and plasma preparation, must be avoided.
 - Discard any plasma presenting an unusual aspect.
 - In order to get the optimal assay performances, the working instructions must be carefully observed.

VARIANT PROTOCOL:

If a higher working range for Rivaroxaban (or other DiXal) is required, the standard assay dilution (d=1:20) can be adjusted accordingly, considering the linearity zone for the assayed DiXal. For example, use a 1:40 dilution (ie d:2) for a working range from 0 to 1µg/ml of Rivaroxaban in the tested specimen. The concentrations measured must then be multiplied by the complementary dilution factor (ie x2 for 1:40 dilution).

USUAL VALUES:

Values described for Xarelto®/Rivaroxaban® in studies on healthy volunteers or on patients with thromboprophylaxis in orthopedic surgery are usually of about 0.1 to 0.2 µg/ml in plasma.

REFERENCES:

- Weitz JI, Hirsh J, Samama MM, American College of Chest Physicians. New antithrombotic drugs : American College of Chest Physicians Evidence-based clinical practice guidelines (8th edition) Chest Suppl. 133(8 suppl):234S-256S ; 2008. Erratum in Chest 2008 Aug; 134(2):473.
- Turpie AGG. New oral anticoagulants in atrial fibrillation. Eur Hart J 29: 155-165; 2007.
- Depasse F, Gerotziolas GT, Busson J, Van Dreden P, Samama MM. Assessment of three chromogenic and one clotting assays for the measurement of synthetic pentasaccharide fondaparinux (Arixtra®) anti-Xa activity. J Thromb Haemost 2: 346-348; 2004 .
- Hirsh J, Dalen JE, Deykin D, Poller L. Heparin : mechanism of action, pharmacokinetics, dosing considerations, monitoring efficacy, and safety. Chest 102: 337S-351S; 1992.
- Perzborn E, Straesburger J, Wilmen A, Pohmann J, Roehrig S, Schlemmer K-H, Straub A. In vitro and in vivo studies of the novel antithrombotic agent BAY 59-7939-an oral, direct Factor Xa inhibitor. J Thromb Haemost 3: 514-521; 2005.
- Kubitza D, Becka M, Volth B, Zuehlendorf M, Wensing G. Safety, pharmacodynamics, and pharmacokinetics of single doses of BAY 59-7939, an oral, direct factor Xa inhibitor. Clin Pharmacol Ther 78: 412-421; 2005.
- Mueck W, Borris LC, Dahl OE, Haas S, Huisman MV, Kakkar AK, Kälebo P, Muelhofer E, Misselwitz F, Eriksson BI. Population pharmacokinetics and pharmacodynamics of once- and twice-daily rivaroxaban for the prevention of venous thromboembolism in patients undergoing total hip replacement. Thromb Haemost 2008 Sep;100(3):453-61.
- Lang D, Weinz C, Schwarz T, Kubitza D, Mueck W. Metabolism and excretion of rivaroxaban - an oral, direct Factor Xa inhibitor - in rats, dogs and humans. Drug Metab Dispos. 2009 Feb 5. [Epub ahead of print]
- http://www.ncbi.nlm.nih.gov; OMIM; "Coagulation factor X" (+227600).