

Specific and Rapid Measurement of Rivaroxaban in Plasma using a New, Dedicated, Chromogenic Assay

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Introduction

Direct Factor Xa Inhibitors such as Rivaroxaban (Xarelto®) have a major therapeutic potential for curative, prophylactic (orthopedic surgery) or long term therapy (as a substitute to dicoumarol type oral anticoagulants).

There is usually no need for patient monitoring during Rivaroxaban therapy at the recommended posology.

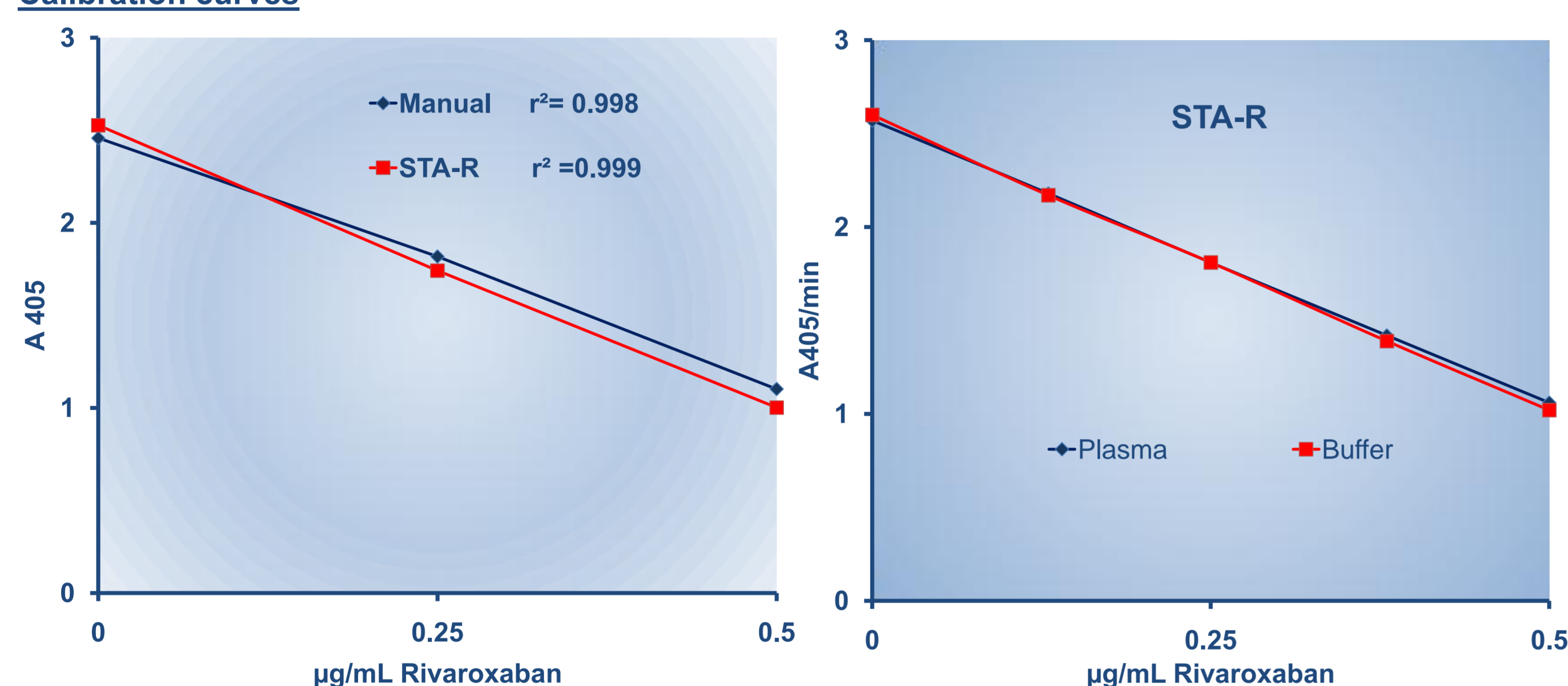
However, drug measurement can be useful in presence of an increased or a decreased drug clearance or when overdosage is suspected. Current anti-Xa heparin assays are not appropriate as they are designed for catalytic indirect Factor Xa inhibitors, requiring Antithrombin for their activity. Furthermore, Factor Xa inhibition kinetics and mechanisms are completely different, which makes necessary using specific Rivaroxaban calibrator and control plasmas. A specific chromogenic assay for measuring Rivaroxaban is presented.

Aim

- ➔ To develop a specific chromogenic assay for measuring Rivaroxaban anti Factor Xa activity in plasma or in any milieu where this drug needs to be measured.
- ➔ To propose specific calibrators and controls for calibration and quality control of the assay, by spiking the drug into normal plasma pool.
- ➔ To validate assay performances in terms of intra- and inter-assay reproducibility, accuracy, limit of quantitation, dynamic range.

Results

Calibration curves

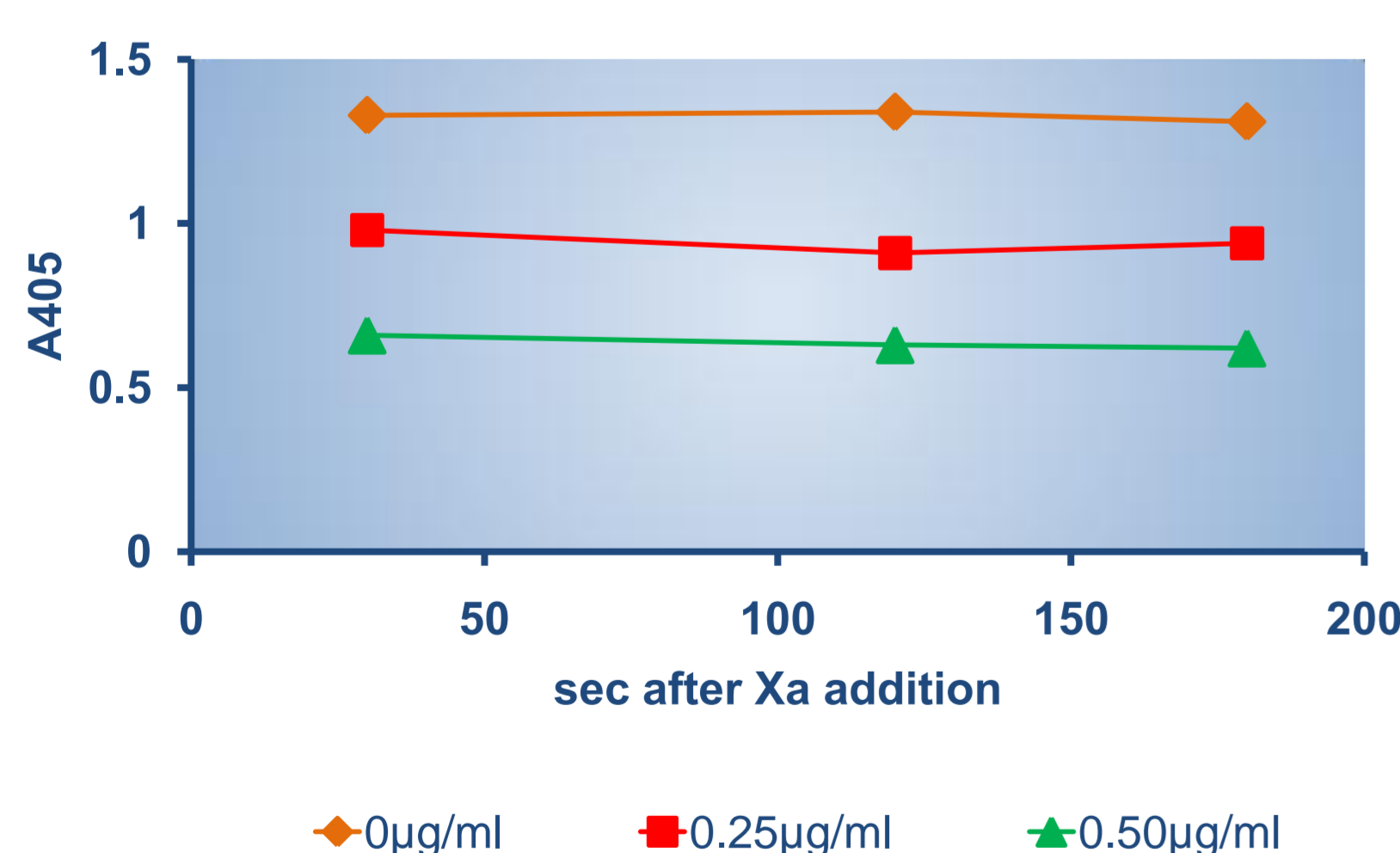


Dose response curves obtained with the manual method or the STA-R automate on the range 0.00 to 0.50 µg/mL.

Calibration curves obtained with Rivaroxaban spiked in plasma or in assay buffer.

- ➔ No matrix effect

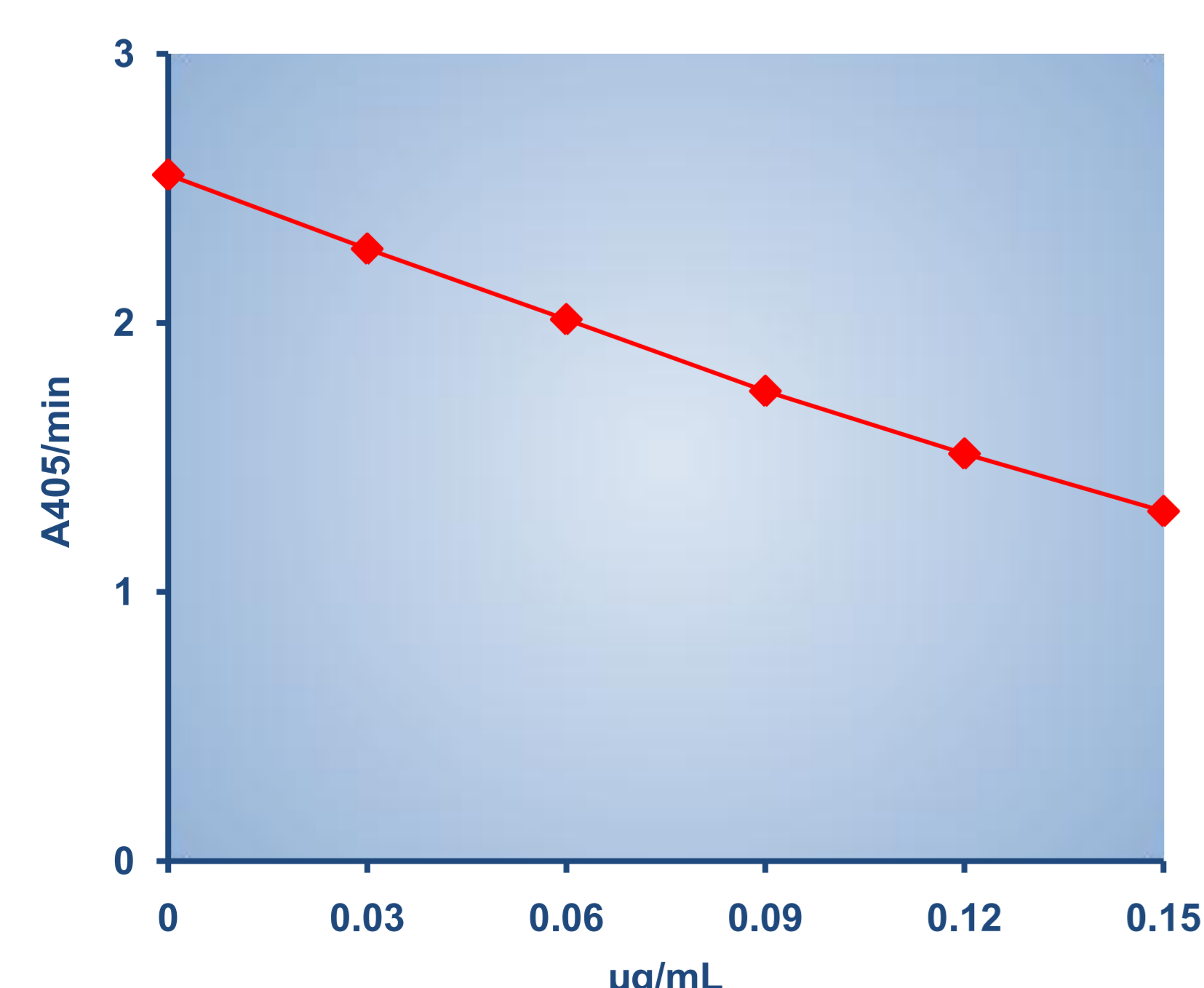
Incidence of the first incubation time following Xa addition (microplate)



- ➔ The incubation time between diluted tested specimen and Factor Xa does not affect the A405 value. There is no "non specific" inhibition of Factor Xa.

- ➔ Factor Xa inhibition is complete within less than 1 minute.

Variant protocol for low range (STA-R)



- ➔ On STA-R the assay working dilution (1:20 instead of 1:50) is easily adjusted to get a low range calibration curve from 0.00 to 0.15 µg/mL (in plasma).

- ➔ This low range calibration curve is useful for measuring Rivaroxaban in outpatients or in prophylaxis. It is also useful when residual amounts of Rivaroxaban need to be evaluated 12 to 24 hours following the last intake.

Assay principle

Two stage chromogenic assay: the diluted tested specimen is first incubated with a constant and in excess concentration of human Factor Xa; Rivaroxaban inhibits human Factor Xa in a mole to mole ratio.

In a second step, residual Factor Xa is tested by its activity on a specific chromogenic substrate.

There is an inverse linear relationship between Rivaroxaban concentration and A405.

Materials and Methods

Rivaroxaban raw material, supplied by Bayer Healthcare, duly characterized and weighted at an exact amount, is diluted first in DMSO, then in a buffer with 5% DMSO and BSA and finally spiked in plasma at the desired concentrations of 0.50 and 0.25 µg/mL.

Tested specimen: Calibrator or assayed specimen are diluted in a high chaotropic buffer preventing heparin-antithrombin interactions (= assay buffer).

Assay range: From 0.00 to 0.50 µg/mL Rivaroxaban in plasma (i.e. 0.00 to 0.025 µg/mL in the assayed dilution).

Protocol

- ➔ Calibrators or controls are assayed diluted 1:20 with assay buffer, or diluted 1:50 for the STA-R application.
 - ➔ **Assay:** 50 µL of specimen are incubated with 50 µL of human Factor Xa (at about 5 µg/mL) for 1 min. at 37°C, then 50 µL of the Factor Xa substrate (CS-11(65)) are introduced and color development is recorded for 45 sec.
 - ➔ Reaction is stopped with 50 µL of 2% citric acid and color is measured at 405 nm.
- The assay can be automated onto any analyzer available in clinical laboratories.

Performance characteristics of the assay

Intra/Inter-assay on STA-R

| Rivaroxaban level (µg/mL) | Intra-assay CV (%) | Inter-assay CV (%) |
|---------------------------|--------------------|--------------------|
| N | 10 | 6 |
| 0.10 | 6.9 | 7.2 |
| 0.30 | 4.3 | 4.1 |

- **Detection threshold**
0.001 µg/mL in the tested milieu

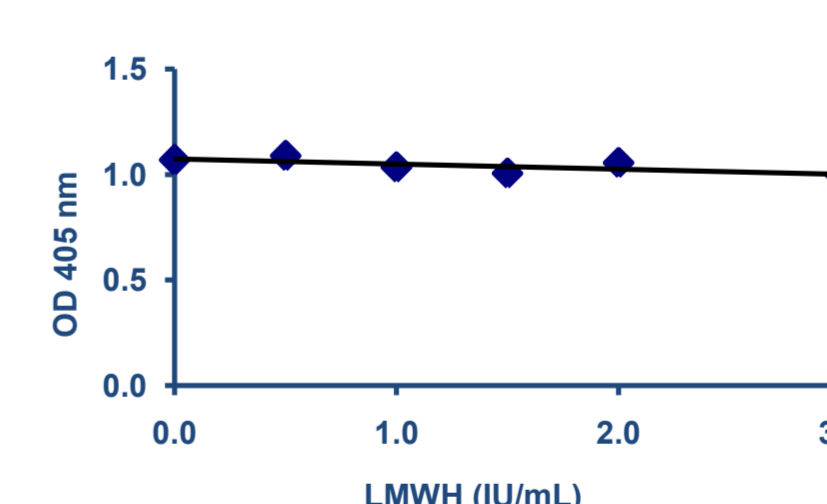
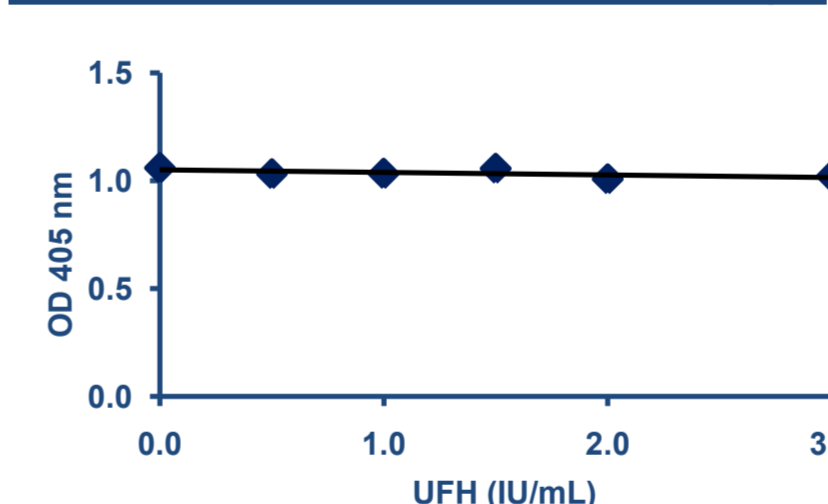
Recovery (N=10) on STA-R

| Plasma spiked with | 0.25 µg/ml | +0,5 µg/ml |
|--------------------|------------|------------|
| Mean µg/mL | 0.24 | 0.47 |
| SD | 0.011 | 0.017 |
| Recovery | 96% | 94% |

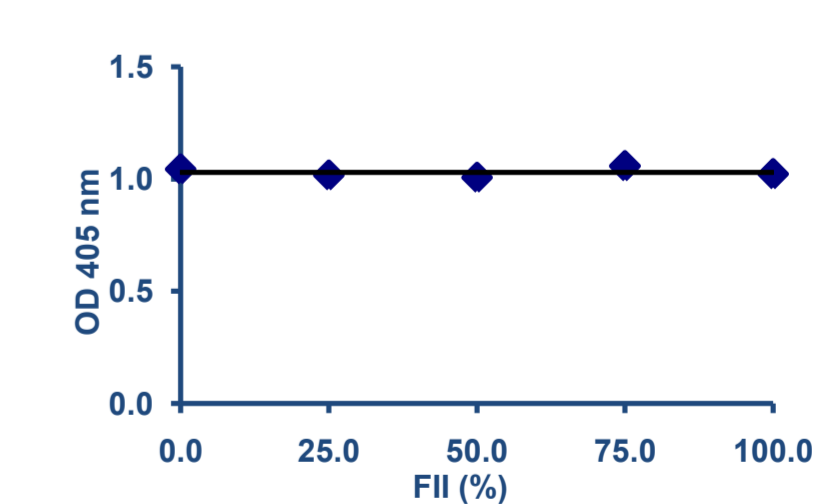
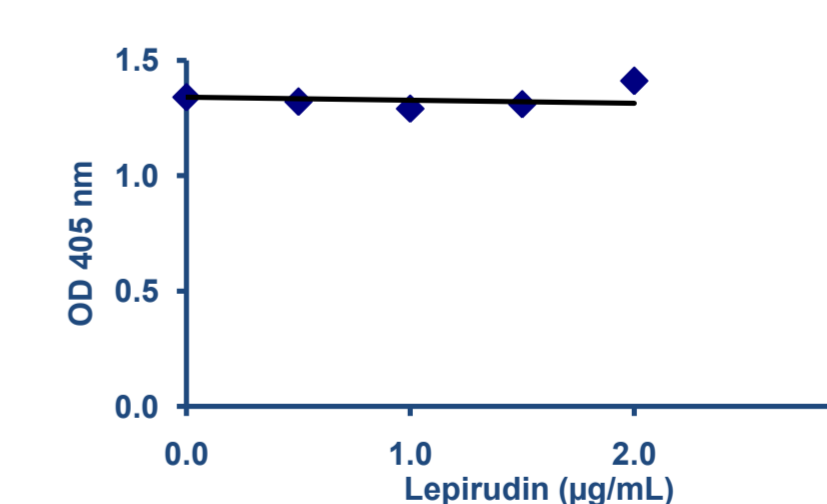
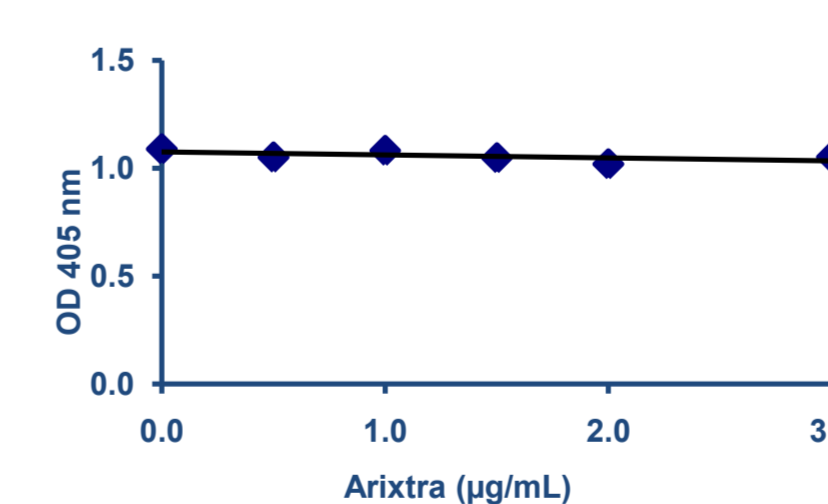
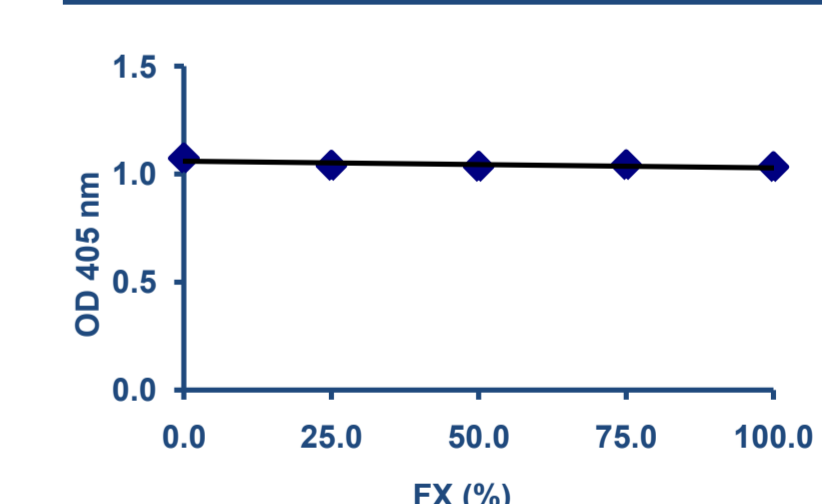
Normal plasmas

N: 33
A405 (STA-R): 2.54 (SD= 0.03)
[C]: ≤ 0.02 µg/mL in plasma (min 0.00 – max 0.02 µg/mL)
Limit of Q: 0.02 µg/mL in plasma

Interference of other drugs



Factor X and Prothrombin



No interference for UFH, LMWH, Arixtra or DTIs, neither of Factor X and Factor II deficiencies.

Conclusions

- ➔ The assay offers a dynamic range from 0.000 to 0.025 µg/mL of Rivaroxaban in the assayed dilution, or from 0.02 to 0.50 µg/mL in plasma.
- ➔ For the expected therapeutic concentrations, plasmas are assayed diluted 1:20.
- ➔ Rivaroxaban recovery is identical whether spiked in assay buffer or in plasma. There is no matrix effect.
- ➔ The method is highly robust, has an inverse and linear dose response relationship ($r^2 > 0.999$), is highly reproducible from run to run (precalibration possible with only 3 concentrations: 0.00, 0.25 and 0.50 µg/mL), without any incidence of the duration of the first incubation time and without matrix effect.
- ➔ Recovery is close to 100 % in plasma, and no protein interference is evidenced. LLOQ is of 0.020 µg/mL in plasma (and 0.001 µg/mL in the assayed dilution).
- ➔ The assay is insensitive to presence of Heparins, Fondaparinux, Lepirudin or to Factor II or X deficiencies.
- ➔ This new simple assay, insensitive to heparins, is fully automatable. It offers an original and reliable laboratory method for measuring anti-Xa activity induced by Rivaroxaban.