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Corresponding Author:
Dr. Juergen Roemisch,
Senior Vice President R&D Plasma, Octapharma PPGmbH, Research & Development, Oberlaaer Strasse 235, 1100 - Austria

Submitting Author:
Dr. Juergen R Roemisch,
Senior Vice President Plasma R&D, Research & Development Octapharma PPGmbH, 1100 - Austria

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Comparison of Thrombin Generation Assay (TGA) and Non-Activated Partial Thromboplastin Time (NAPTT) for the Assessment of Enhanced Procoagulant Activity in Immunoglobulin Solutions

Author(s): Roemisch J, Zapfl C, Zoechling A, Pock K

Abstract

The recent revision of the European Pharmacopoeia stipulates that all intravenous immunoglobulin solutions must be investigated with respect to procoagulant activity. Accordingly, the manufacturing processes must be validated regarding the removal of such compounds, to ensure a minimized thromboembolic risk for patients. Furthermore, based on recent studies, which identified the presence of occasionally elevated concentrations of activated factor XI (FXIa) in an immunoglobulin solution as root cause for thromboembolic events in patients, a thrombin generation assay (TGA) was validated and is routinely performed for Octagam® batch release.

Non-activated partial thromboplastin time (NAPTT) is discussed as an alternative to TGA, because it is a suitable and wide-spread method to assess an enhanced procoagulant potential of coagulation factor concentrates. However, in order to meet the sensitivity provided by the validated TGA and its defined cut-off limits for Octagam® batch release, the corresponding limits of NAPTT would require a new definition to ensure a comparable safety margin.

This means that under the usually performed NAPTT conditions several of those batches with a moderately increased procoagulant activity would be missed, which however would be identified by the implemented TGA criteria. Consequently, NAPTT might become an alternative only, if validated against TGA and its defined sensitive cut-off limits. This would include revision of generally communicated NAPTT assay protocols.

As reported for TGA, factor XI deficient plasma (FXI-DP) revealed improved NAPTT performance when compared to fresh-frozen plasma or pooled normal plasma. This report also demonstrates that traces of other activated factors (FIXa and FXa) were detected in FXI-DP with high sensitivity.

In conclusion, TGA with FXI-DP is the preferred assay for the exclusion of immunoglobulin solution batches with increased procoagulant potential. TGA is superior to established NAPTT performance.

Introduction

In the period of 2010-2011 two intravenous immunoglobulin solutions (IVIG) were recalled from the markets because of thromboembolic events (TEEs), and a risk for thrombotic adverse events was reported for a subcutaneous immunoglobulin concentrate (1-5). This initiated a discussion about the root cause(s) and the assessment of sensitive and robust assays to detect present compound(s) enhancing the procoagulant activity in immunoglobulin preparations (6-11). Occasionally Increased concentrations of activated FXI (FXIa) in some Octagam® batches were identified as the biochemical root cause, which correlated with parameters of a thrombin generation assay (TGA), namely elevated peak thrombin concentration (PTC), shorter lag time and time to peak (TTP) (1,12).

Consequently, corrective and preventive measures were implemented into the Octagam® manufacturing process. The Technothrombin® based TGA was established and validated by Octapharma´s Quality Control department for Octagam® batch release (1, 12). A large safety margin is provided by the defined specifications based on the use of FXI deficient plasma (FXI-DP) for TGA.

After validation with the defined cut-off limits TGA was approved by regulatory authorities for Octagam® batch release. However, the discussion about potential alternatives to TGA for this application continued, in particular because a recent revision of the European Pharmocopoeia (Ph. Eur.) stipulates process validation for all IVIG products regarding a robust removal of potentially present procoagulant substances (13).

Non-activated partial thromboplastin time (NAPTT) is a coagulation assay, which is known to be sensitive to the presence of activated coagulation factors. It is a part of the Ph. Eur. (2.6.22), performed for the release

of for instance FIX concentrates, but not yet for immunoglobulin solutions. In particular the required sensitivity and cut-off limits are not yet defined for other product groups than those mentioned above. Further investigations are required to gain information how cut-off limits of the different assays would relate to each other, taking into account potential matrix effects of different immunoglobulin solutions.

We compared the implemented TGA in particular with NAPTT regarding their usefulness to identify an enhanced procoagulant activity in immunoglobulin solutions. For this purpose we used historical Octagam® batches with elevated FXIa contents and tested them in whole plasma samples and FXI-DP. In addition to FXIa, other proteases were investigated in FXI-DP based TGA and NAPTT.

Material and Methods

Material

Pooled normal plasma (PNP) and FXI deficient plasma (FXI-DP) were from Precision Biologics LLC (USA). Activated proteases for spiking experiments were obtained from the following sources: FVIIa was the 2nd International Standard FVIIa concentrate from NIBSC (UK), purified human factors FIXa and FXa from Hyphen BioMed (France), human FXIa from Enzyme Research Labs Inc. (USA) and Haematologic Technologies Inc. (USA), recombinant human plasma Kallikrein from R&D Systems Inc. (USA) and FXIIa, prekallikrein activator (PKA) in albumin BRP batch 2 from EDQM (France). Recently produced FXIa-free and historical Octagam® batches, the latter of which contained various concentrations of FXIa, were investigated by the different coagulation assays. The platelet poor plasma (PPP) was prepared from freshly drawn citrated blood samples as supernatant obtained after centrifugation for 20 min at 4°C and 1,962xg. Single donor fresh-frozen plasma (FFP) samples were used immediately after complete thawing.

Analytical Methods

TGA

Immunoglobulin, e.g. Octagam®, was mixed with standard human plasma or FXI-DP to obtain a final concentration of 1 % IgG. The thrombin generation was measured using the Technothrombin® TGA calibration set, substrate and the RC high trigger from Technoclone GmbH (Austria) as well as a Fluostar Optima (BMG Labtech GmbH, Germany) and the TGA evaluation software from Technoclone. Different concentrations of activated clotting factors were spiked into the sample prior to addition of TGA reagents. The thrombin concentration of the sample was calculated from the change of fluorescence of the substrate over time using the thrombin calibration curve. The main readout parameters for the samples are lag time (min), TTP (min) and PTC (nM thrombin).

NAPTT

The working dilution of the STA Cephalin (Hoffmann-La Roche AG, Germany) was determined according to Ph. Eur. 2.6.22. In order to adjust clotting times of the plasma samples to a range of 200-350 sec, the reagent was (if required) diluted with water. Octagam® samples or Octagam® mixed with activated factors were pre-diluted with Tris buffer 1:5, 1:10 or 1:100 and mixed with equal amounts of plasma (FXI-DP, PNP, FFP or freshly prepared platelet poor plasmas), STA Cephalin and $CaCl_2$. The time until clot formation was recorded using a BCS XP (Siemens AG, Austria).

ROTEM®

Immunoglobulinsolutions, in particular Octagam® samples, were mixed with plasma to obtain a final concentration of 1% IgG and the clot formation was recorded using a ROTEG 05 thrombelastograph (TEM Innovations GmbH, Germany). The sample was added into a disposable cuvette in a heated cuvette holder. The NATEM test was performed by re-calcification of a plasma sample, which was contact-activated by the surface of the measurement cell. Accordingly, the reaction was initiated by the addition of $CaCl_2$ as provided in the star-TEM test kit (TEM Innovations). The loss of the elasticity due to clot formation led to a change in the rotation of the axis. These data were visualized in thromboelastograms. The read-out parameter was clotting time (CT).

FXIa

FXIa was quantified by a modified FIXa test from Hyphen BioMed as follows: Factor IX (recombinant product Benefix®, Pfizer, Wyeth Pharmaceuticals Inc., USA) was activated by FXIa contained in the test sample. In the presence of phospholipids and calcium FIXa forms an enzyme complex with thrombin-activated FVIII (which is present in excess at a constant concentration). This enzyme complex converts FX (as part of the assay setting) to FXa. The amount of FXa is directly proportional to the amount of FIXa, which represents the limiting factor. The FXIa concentration is calculated from the standard curve obtained with a FXIa reference concentrate (Enzyme Research Labs Inc., USA).

Results

Impact of sample pre-dilution on NAPTT

Prior to comparison of NAPTT with other methods, the impact of sample pre-dilution was tested. Increasing concentrations of FXIa were spiked into an Octagam® sample, which was free from traces of FXIa. NAPTTs were determined using FXI-DP after mixing with the FXIa containing Octagam® samples at different ratios. A comparable sensitivity to detect FXIa contents was found for the ratios 1:1, 1:5 and 1:10 (pre-dilution), but was lower for 1:100 (Illustration 1). Consequently, the following NAPTT measurements were performed at ratios of 1:5 and/or 1:10. Use of FXI-DP revealed high sensitivity to FXIa even at concentrations below 1mU/ml.

Comparative analyses using historical Octagam® samples containing FXIa

TGA and NAPTT

Octagam® batches (historical retention samples) containing different concentrations of FXIa were analyzed in the different assays described below. These batches covered a wide range of *in vitro* procoagulant activity as identified by TGA and NATEM. Illustration 2A shows NAPTT (1:10) and TGA parameters tested in PNP, whereas Illustration 2B refers to FXI-DP. A clear correlation between NAPTT and TGA values was confirmed using both, PNP and FXI-DP, while the latter offered extended measurement ranges in both assays. The correlation between NAPTT and TGA parameters was better using FXI-DP. Tentative NAPTT cut-off values (sec) were calculated referring to the TGA parameters listed in Illustration 3.

Compared to the tentative cut-off limits for NAPTT (PNP: 414 sec, FXI-DP: 450 sec) referring to PTC values in Illustration 1, a considerably lower number of batches would be excluded by NAPTT cut-off limits as communicated for coagulation products (150-200 sec) (Illustration 4).

Correlation of FXIa concentrations with TGA, NAPTT and NATEM using FXI-DP

In the same Octagam® samples as described above, the FXIa contents were quantified. In addition to TGA and NAPTT, NATEM was performed in FXI-DP. The results are shown in Illustrations 5 and 6. Correlations were found between the coagulation times in the different assays, i.e. TGA/TTP, NAPTT and with NATEM clotting times (19 min TTP matched with 908 sec NATEM). The FXIa content had a considerable impact on clotting times and thrombin generation potential, even below 1 mU/ml. A PTC of 350 nM correlated with a FXIa concentration around 1 mU/ml. Corresponding clotting times of ?400-450 sec in NAPTT using FXI-DP would exclude significant FXIa contents.

Single donor PPP: NAPTT and TGA

Ph. Eur. recommends adjustment of the neat plasma sample to a clotting time range of 200-350 sec, by optimal mixing of the (pre-diluted) assay reagent. As this was sometimes hardly possible with PNP (even with non-diluted assay reagent), we investigated platelet poor plasma (PPP) prepared from freshly drawn single blood donations, which were mixed with different dilutions of the test reagent. Having defined the individually required reagent concentration, the PPP was spiked with increasing amounts of FXIa and NAPTTs were measured. Illustration 7 shows the shortening of clotting times of two individual PPPs dependent on the FXIa concentration. In addition, the PPPs were mixed with samples of the Octagam® batches described above and NAPTTs (1:10) and TGA parameters were determined. Illustration 8 presents the correlation between NAPTT, NATEM and TGA parameters in both PPPs. Considerable inter-individual differences of the NAPTT/TGA characteristics were observed, if reagent dilutions were used after individual adjustment of the NAPTTs into the 200-350 sec target range. Therefore, based on individual PPP it is hardly possible to define an absolute NAPTT cut-off limit for the exclusion of a batch with enhanced procoagulant activity. Even a ratio of neat/sample would not provide sufficient information due to the adjusted NAPTT range and the high inter-individual variation. The corresponding individual NAPTT cut-off limits would be 167 sec for PPP1 (donor 1) and 300 sec for PPP2 (donor 2) referring to a TGA/PTC of 350 nM.

The number of procoagulant batches which would not be identified by fixed NAPTT cut-off levels of 150 or 200 sec are listed in Illustration 9 (corresponding numbers relating to a PTC of 350 nM are added for reference). The results demonstrate that the number of excluded batches with an enhanced procoagulant activity would vary considerably if based on single donor PPP. Notably, based on PPP2, a NAPTT cut-off limit of 150 sec would not have identified a single batch with enhanced procoagulant activity as compared to a TGA/PTC of 350 nM. Choosing a cut-off of 200 sec would increase the number of excluded batches, but differently for PPP1 and PPP2.

As a consequence, individual PPPs cannot be recommended for the assessment of batch release due to too high variability of baseline levels and individual responses. This might be overcome by

pooling of freshly prepared PPPs (usually at least 30 single donations are pooled, example PNP) or separate testing of a representative number of individual PPPs. Both options would be impractical and would cause too high variation.

Impact of spiked FXIa on NAPTT (adjusted), performed with individual PPPs

Both PPPs were spiked with increasing concentrations of FXIa, after individual adjustment into a NAPTT starting range of 200-350 sec. For comparison, the same plasma samples were spiked with FXIa and PTC was measured by TGA. The sensitivity to differentiate FXIa/procoagulant levels differed considerably regarding both assays. In summary, a PTC of 350 nM was more sensitive to FXIa than the NAPTT of 150-200 sec, as illustrated in Illustration 10. The red arrow demonstrates the PPP dependent differences on the 350 nM PTC/TGA level, whereas the blue rhombus shows the area of 150 sec and 200 sec NAPTT for both PPPs.

Impact of different activated factors on NAPTT and TGA using FXI-DP

NAPTT: An Octagam® sample free from procoagulant activity, i.e. no activated factors detectable, was spiked with commercially available activated and activity assigned proteases of the coagulation and contact phase systems, namely FVIIa, FIXa, FXa, FXIa, FXIIa (PKA) and Kallikrein. Using FXI-DP, the NAPTT could not be adjusted into a target range (200-350 sec) prior to spiking. NAPTTs were measured after mixing with the spiked Octagam® samples. The *in vitro* dose response curves on NAPTT are presented in Illustration 11. FXIa had the strongest impact on shortening of NAPTT at lowest protease concentrations, whereas the relative effect of FIXa and FXa became more pronounced with increasing concentrations (steepest curves). The impact of FVIIa, FXIIa and Kallikrein was negligible at low concentration.

TGA: Similar to NAPTT, TGA parameters (PTC, lag time, TTP) were most sensitive to FXIa (Illustration 12-14). FVIIa and FXIIa had a negligible effect under these conditions, but Kallikrein caused a small to moderate increase of PTC (Illustration 12) in a concentration range of 1-10 µg/ml.

In summary, TGA and NAPTT (and NATEM) in principle were suitable to detect the presence of activated factors spiked into an Octagam® sample, while the use of FXI-DP offered a broader measurement range for these assays. In addition, NAPTT as described in the Ph.Eur. (adjusted neat of 200-350 sec) was reproducibly achieved with freshly prepared PPP (at individual pre-dilutions of cephalin reagent), but was not always possible with PNP or FFPs (latter not shown). Independent of the plasma preparation used, the defined TGA criteria described were more sensitive than the established NAPTT cut-offs for the detection of procoagulant factors in coagulation concentrates.

Discussion

The analyses confirmed that NAPTT is in principle suitable to detect procoagulant activity in Octagam® samples, but the comparability between NAPTT and the TGA cut-off limits for the batch release of Octagam® deserved more detailed assessment.

NAPTT based investigation of coagulation concentrates such as FIX concentrates starts at a clotting time (adjusted) in the range of 200-350 seconds. A shortening of NAPTT to ≤150 seconds caused by the test sample is regarded as indicative for a relevant enhanced procoagulant activity. Individual PPPs met the principle requirement by Ph.Eur., thus could be adjusted to a starting range of 200-350 sec NAPTT, but needed individual pre-dilution of the assay reagent to reach this range. Therefore, PPPs can respond very differently after spiking. This is a reason to use pooled plasma, which levels out individual differences. In case of PNP at least 30 PPPs are usually pooled and distributed as frozen aliquots. Notably, PNP could not be brought reproducibly into the required 200-350 sec NAPTT range by using commercially available reagents (other providers not yet investigated). This causes the dilemma of either using individual PPPs, which can hardly be compared and are difficult to standardize, or PNP, which would not start at the recommended levels (or at the upper borderline). This would be a considerable disadvantage in defining cut-off levels for the identification of immunoglobulin batches with increased procoagulant activity.

Furthermore, independent of the plasma preparation used in this study, TGA cut-off levels were more sensitive than the currently discussed NAPTT exclusion ranges for coagulation products (≤150 sec). This means, a procoagulant sample, if mixed with complete human plasma (either PNP, FFP or PPP), would not even come close to the safety margin achieved by the TGA specifications for Octagam® batch release. A NAPTT of around 200 sec might have identified Octagam® batches which were associated with TEEs, containing more than 17 mU of FXIa/ml (1). Studies in the Wessler stasis model had demonstrated that batches containing >7 mU FXIa/ml caused

moderate thrombus formation in rabbits (1). This FXIa concentration might have resulted in a NAPTT around 200 sec. However, this would not provide a safety margin on the same level achieved by the defined TGA cut-off limits.

When using cut-offs of 150 or 200 seconds, a number of historical Octagam® batches with enhanced procoagulant activity, as determined by TGA, would not have been identified by NAPTT. Therefore, either a NAPTT ratio (neat/sample) would have to be validated or an absolute coagulation time against the current TGA parameters would have to be established in order to provide a comparable safety margin. The results reported would clearly favour the use of whole plasma without prior adjustment or FXI-DP for the performance of NAPTT in order to increase the sensitivity of the method. As different immunoglobulin solutions have different formulations/matrices, TGA or NAPTT must be validated for each product to demonstrate a corresponding safety margin, considering also other or additional procoagulant compounds than FXIa. Certainly, the specificities of different TGAs must be taken into consideration if validating processes and specifications for other products. A collaborative study is ongoing to evaluate the characteristics of different assay methods and individual test performance and reagents.

The use of FXI-DP is discussed in terms of limiting analyses to identify procoagulant factors other or in addition to FXIa. In order not to bias an objective analytical assessment towards FXIa, initially whole plasma was used in order not to miss important procoagulant components. After identification of the root cause in TEE-associated Octagam® batches, namely FXIa, the assay was optimized using FXI-DP achieving a wider measurement range. In principle other activated factors, namely FIXa, FXa and Kallikrein (at high concentrations) can also be detected (after spiking) in FXI-DP.

Alternative approaches to perform NAPTT or TGA (TGT) methods for the characterization and identification of IVIG batches with enhanced procoagulant activity were discussed, for instance based on corn trypsin inhibitor (CTI) plasma, but this requires more detailed investigation, because other characteristics and limitations have to be considered (6).

Consequently, for other immunoglobulin solutions it must be validated which assay is best suited to facilitate the definition of a sufficient safety margin. NAPTTs performed for coagulation products with commonly used cut-off limits (150-200 seconds) are less sensitive than the reported TGA limits, which provide a large safety margin. Consequently, revision of established NAPTT clotting times or ratios would be required before used for the batch release of immunoglobulin solutions.

Abbreviations

APTT- Activated Partial Thromboplastin Time
CTI- Corn Trypsin Inhibitor
DP- Deficient Plasma
EDQM- European Directorate for the Quality of Medicines & Healthcare
ELISA- Enzyme Linked ImmunoSorbent Assay
FFP- Fresh-Frozen Plasma
FVIIa- Factor FVII activated
FIXa- Factor IX activated
FXa- Factor X activated
FXIa- Factor XI activated
FXIIa- Factor XII activated
IVIG- Intravenous Immunoglobulin
Min- minutes
ml- milliliter
mU- milliunit
NAPTT- Non-Activated Partial Thromboplastin Time
nM- nano-Molar
Ph. Eur.- European Pharmacopoeia
PKA- PreKallikrein Activator
PNP- Pooled Normal Plasma
PPP- Platelet Poor Plasma
PTC- Peak Thrombin Concentration
QC- Quality Control
ROTEM®- Rotational Thrombelastometry
sec- seconds
TEE- Thromboembolic Event
TGA- Thrombin Generation Assay
TGT- Thrombin Generation Test
TTP- Time To Peak

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Illustrations

Illustration 1

NAPTTs of FXI-DP mixed with Octagam samples (spiked with FXIa) at different ratios. FXIa levels refer to final concentrations in spiked Octagam.

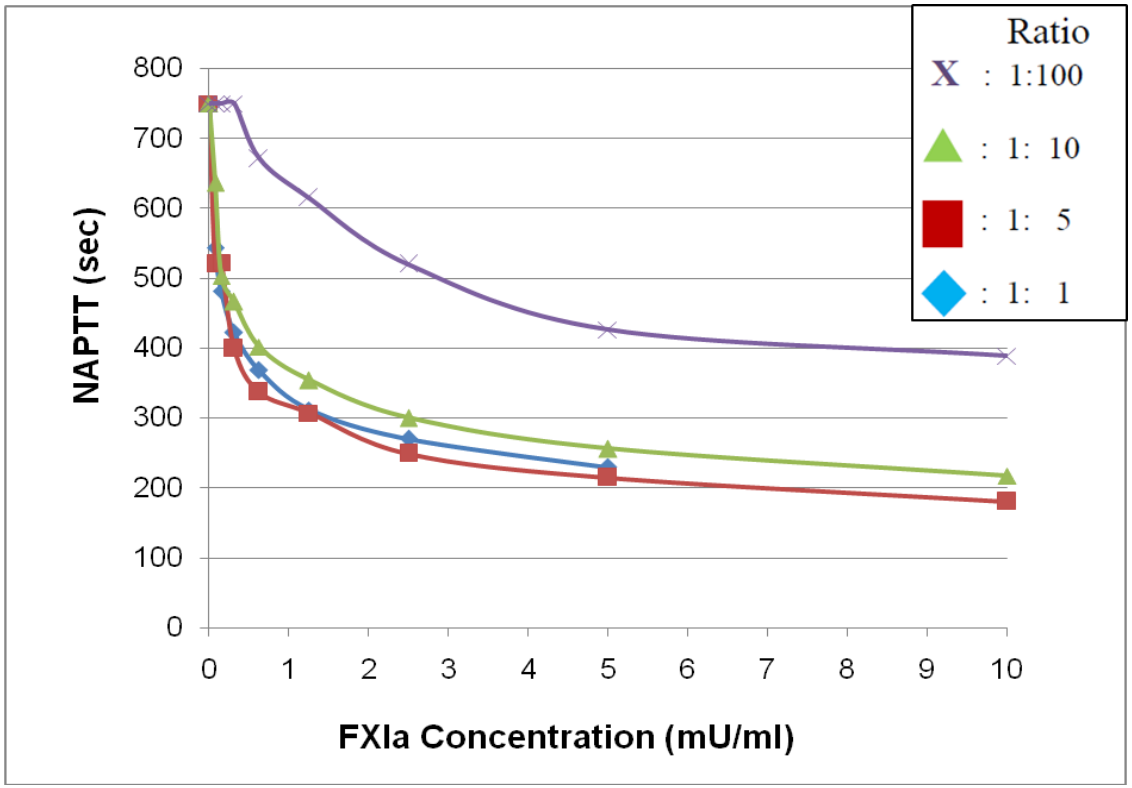

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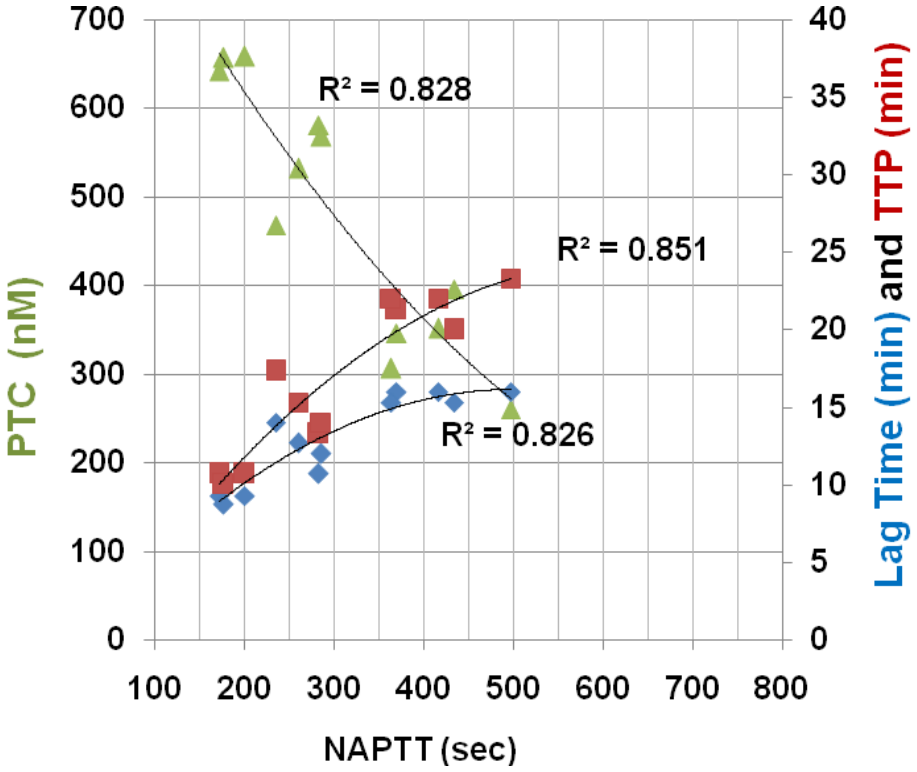
Illustration 2

FXIa containing Octagam batches tested in (A) PNP and (B) FXI-DP based assays. Data points depict the characteristics of different historical Octagam batches (n=12).

A



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B

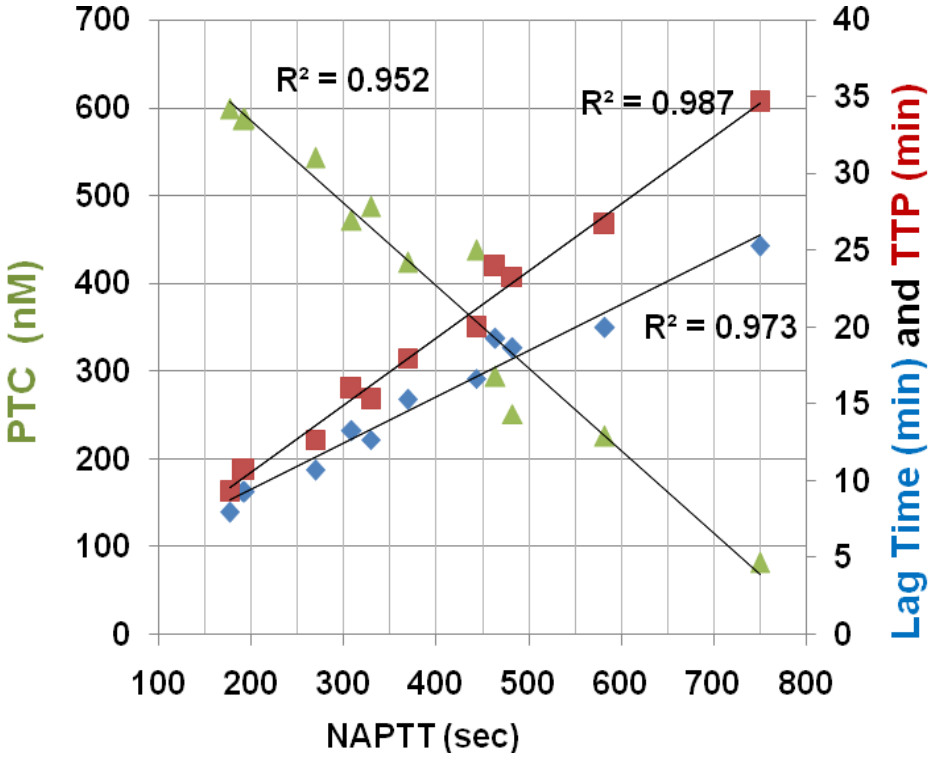

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Illustration 3

Tentative cut-off limits for NAPTT (sec) referring to PTC (350 nM)

TGA Parameter	Corresponding NAPTT (PNP; in sec)	Corresponding NAPTT (FXI-DP; in sec)
PTC: 350 nM	414	450
Lag Time: 11 min	226	250
TTP: 19 min	363	392

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Illustration 4

Number of batches identified based on different NAPTT cut-off limits. Tentative NAPTT cut-off limits for PNP and FXI-DP corresponding to a TGA/PTC (350 nM, Illustration 3) were used to calculate the number of excluded historical Octagam batches containing different concentrations of FXIa. In addition, NAPTT cut-offs of 150 sec and 200 sec were evaluated.

NAPTT cut-off limit (sec)	PNP based: excluded / total	FXI-DP based: excluded / total
≤ 414 (PNP); ≤ 450 (FXI-DP)	9 / 12	9 / 12
≤ 200	2 / 12	3 / 12
≤ 150	0 / 12	0 / 12
TGA / PTC : 350 nM	9 / 12	8 / 12

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Illustration 5

Correlation of TGA/PTC with NAPTT and NATEM. FXIa containing Octagam batches were tested after spiking into FXI-DP. Coagulation times (NAPTT and NATEM in sec) and PTC (TGA, nM) were determined. Corresponding FXIa concentrations were quantified as described under Material and Methods. Open squares indicate FXIa concentrations < 0.32 mU/ml.

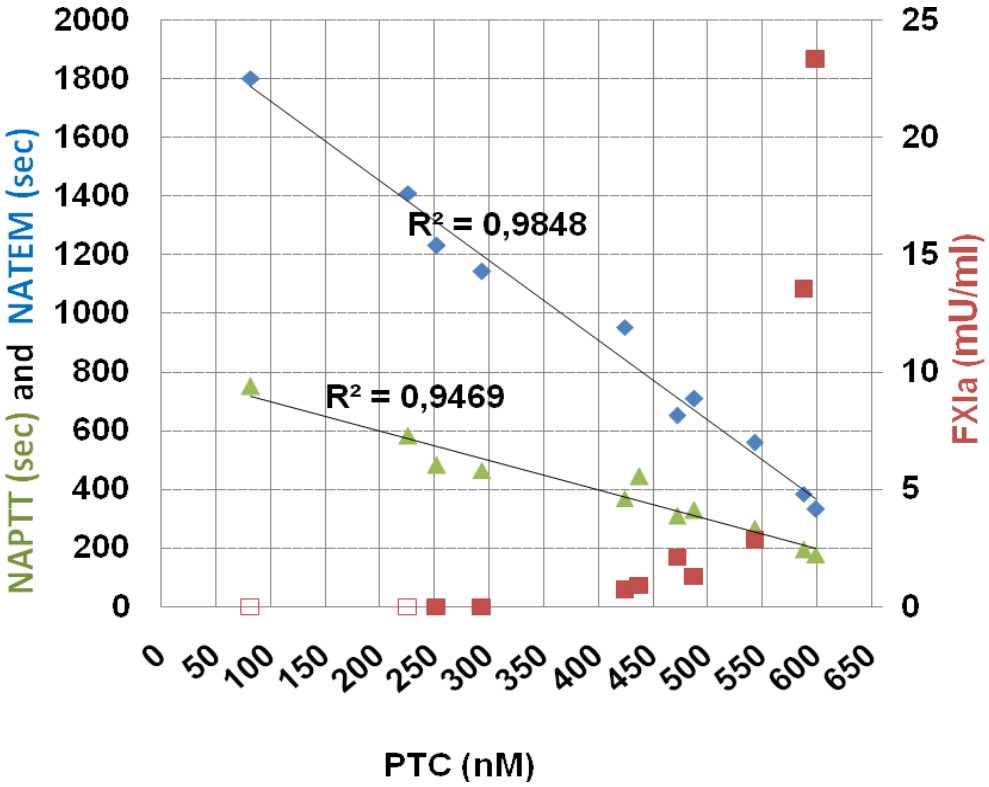

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Illustration 6

Correlation of TGA/TTP with NAPTT and NATEM. FXIa containing Octagam batches were tested after spiking into FXI-DP. Coagulation times (NAPTT and NATEM in sec) and TTP (TGA, sec) were determined. Corresponding FXIa concentrations were quantified as described under Material and Methods. Open squares indicate FXIa concentrations < 0.32 mU/ml.

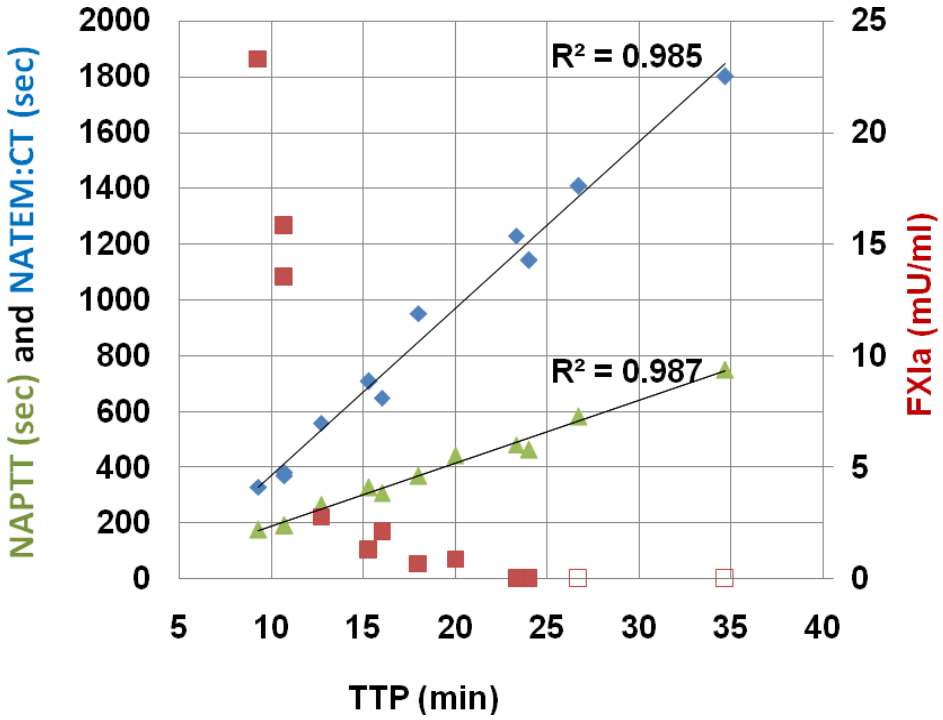

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Illustration 7

NAPTTs of two freshly prepared PPPs spiked with FXIa. PPPs were adjusted by assay reagent (undiluted or 1:8 diluted, respectively) to clotting times in the range of 200-350 seconds prior to spiking with FXIa. FXIa levels refer to final concentrations in each PPP.

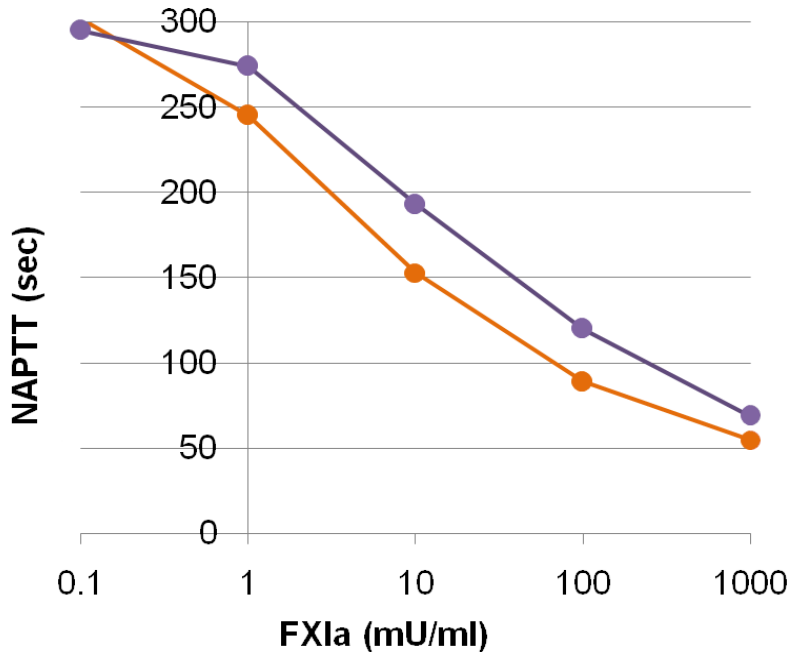

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Illustration 8

8

Correlation of NAPTTs (start: 200-350 sec) with TGA/PTCs (n=15) and with NATEM (n=6) after addition of historical Octagam® batches. PPPs of two donors are presented (PPP 1: squares; PPP 2: diamonds).

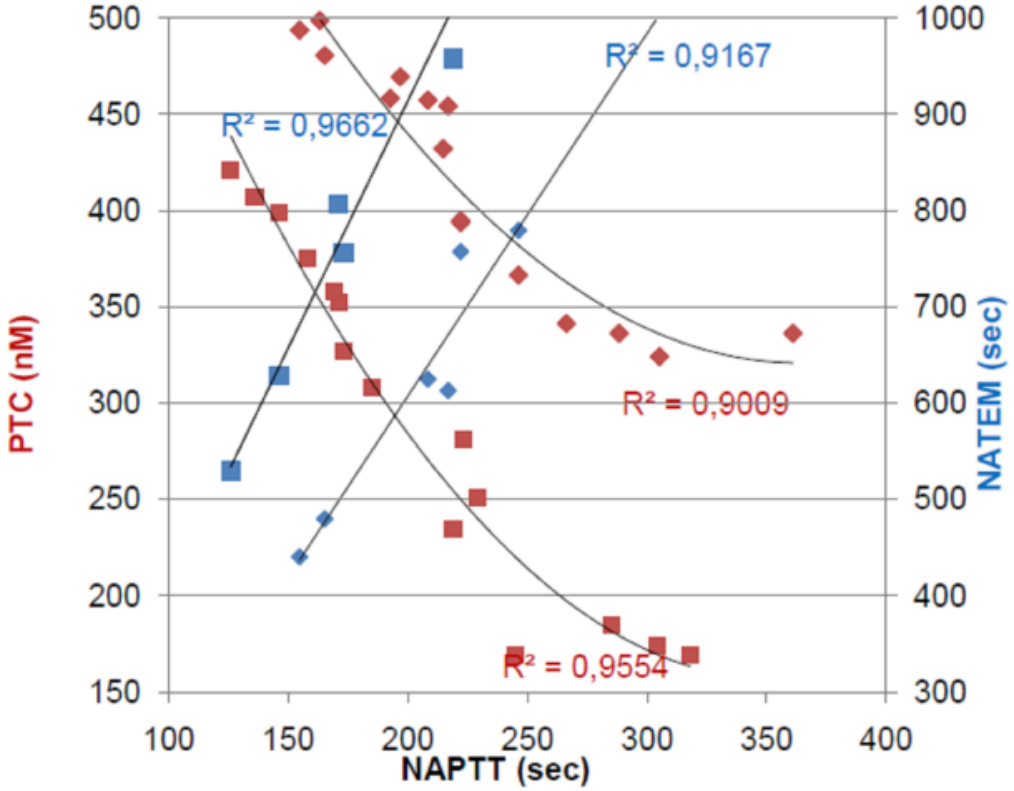

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Illustration 9

PPP based identification of batches with increased procoagulant activity using TGA/PTC (350 nM) and NAPTT cut-off limits (150 or 200 sec). Two freshly prepared PPPs were used for the identification of batches with increased procoagulant activity. Before mixing with each sample, the individual PPPs were brought into a NAPTT starting range of 200-350 sec.

NAPTT cut-off limits or TGA	**PPP 1: excluded/total**	**PPP 2: excluded/total**
NAPTT: ≤ **150 seconds**	4 / 15	**0** / 15
NAPTT: ≤ **200 seconds**	8 / 15	**3** / 15
TGA PTC : **> 350 nM**	6 / 15	**10** / 15

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Illustration 10

NAPTTs and TGA/PTCs of two PPPs spiked with FXIa. NAPTTs are indicated as squares, TGA/PTC values as circles; PPP1: orange characters; PPP2: blue characters. PPPs were mixed with assay reagent at concentrations to achieve a NAPTT clotting time range of 200-350 sec prior to spiking with FXIa. The blue rhombus indicates FXIa concentrations causing a NAPTT in the range of 150-200 seconds. The red arrow shows the > =350 nM TGA/PTC caused by spiked FXIa.

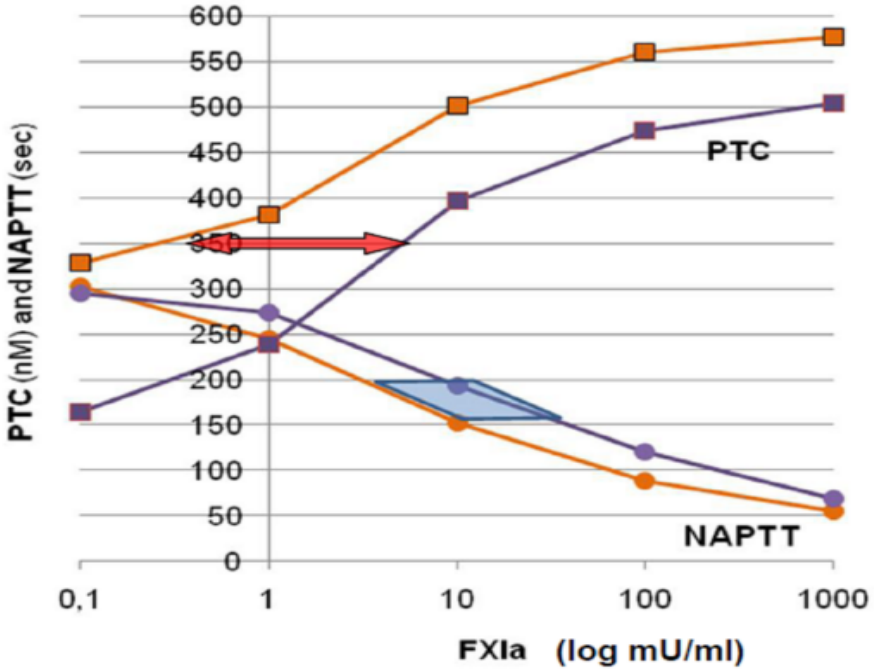

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Illustration 11

11

Impact of activated factors on NAPTT using FXI-DP. An Octagam® sample free from procoagulant potential, i.e. no activated factors were detectable, was spiked with commercially available activated and activity assigned proteases of the coagulation and contact phase systems

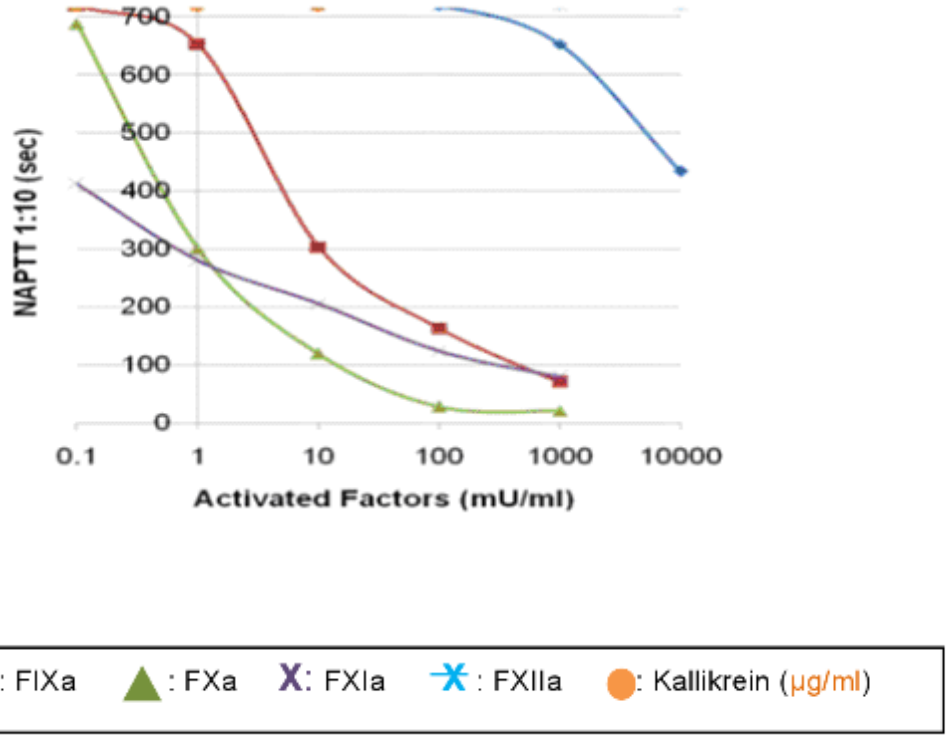

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Illustration 12

Impact of activated factors on TGA/PTC in FXI-DP. An Octagam sample free from procoagulant potential, i.e. no activated factors were detectable, was spiked with commercially available activated and activity assigned proteases of the coagulation and contact phase systems.

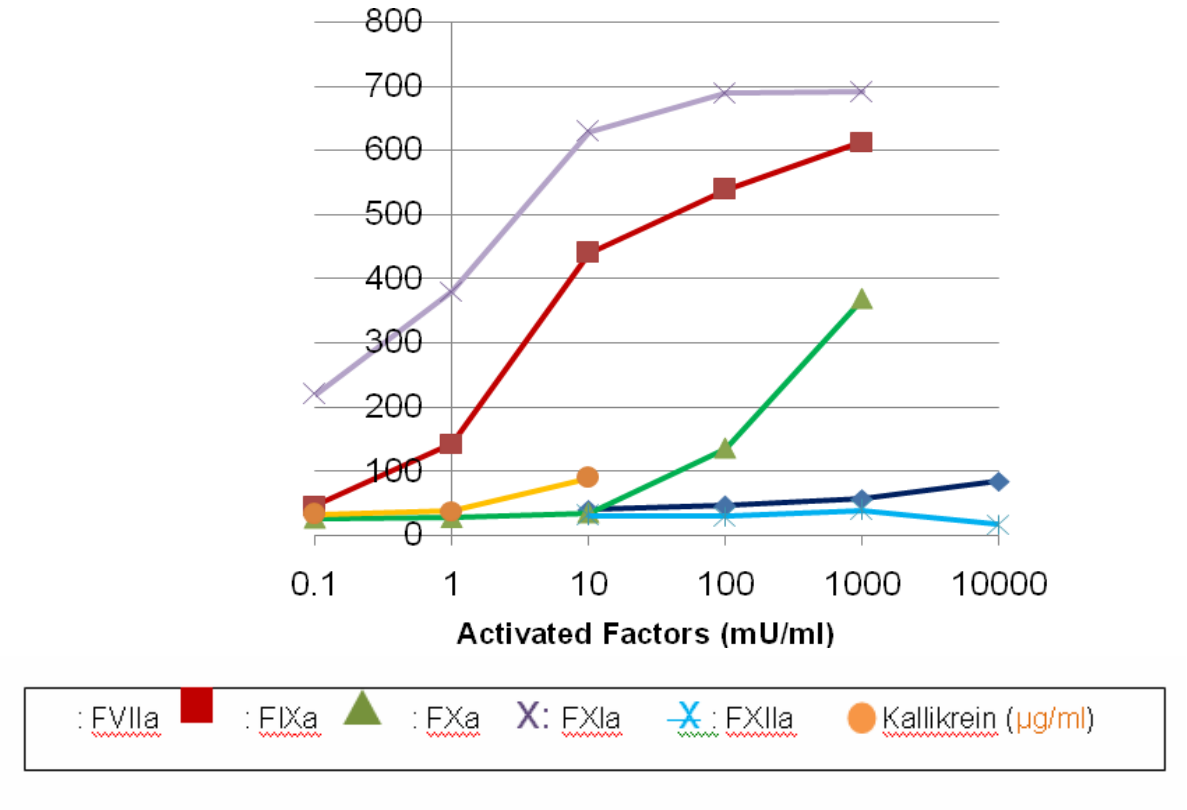

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Illustration 13

Impact of activated factors on TGA/PTC in FXI-DP. An Octagam sample free from procoagulant potential, i.e. no activated factors were detectable, was spiked with commercially available activated and activity assigned proteases of the coagulation and contact phase systems.

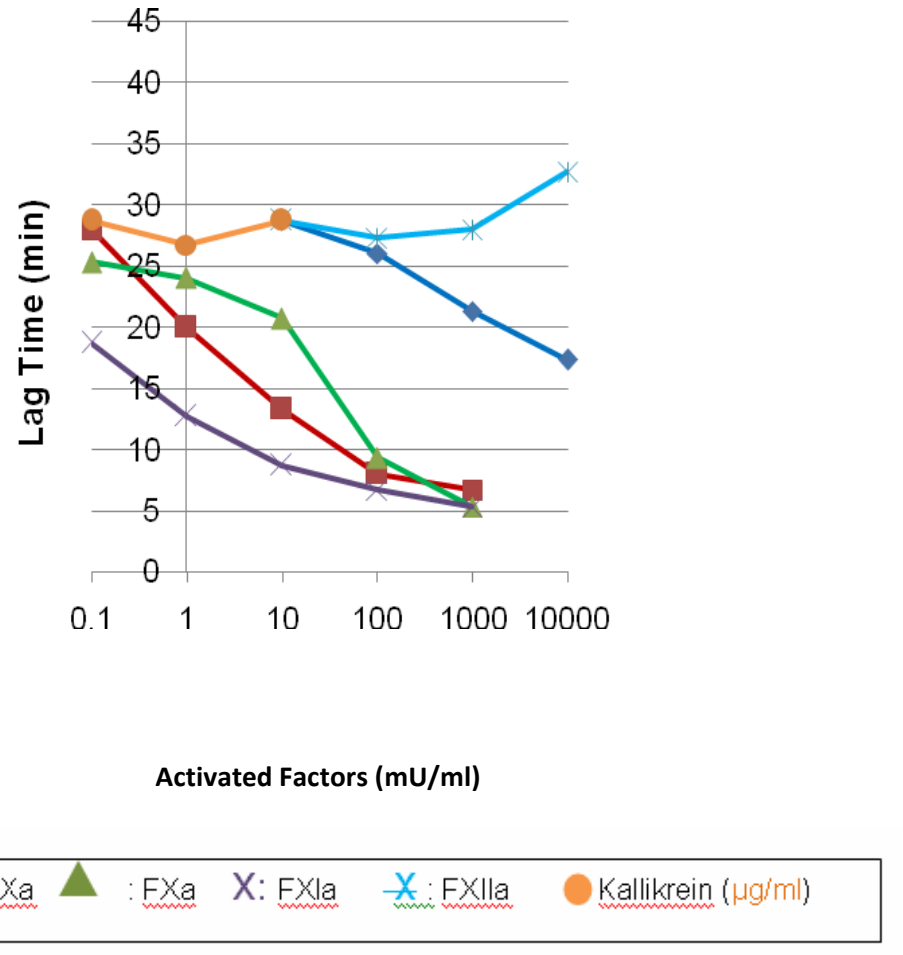

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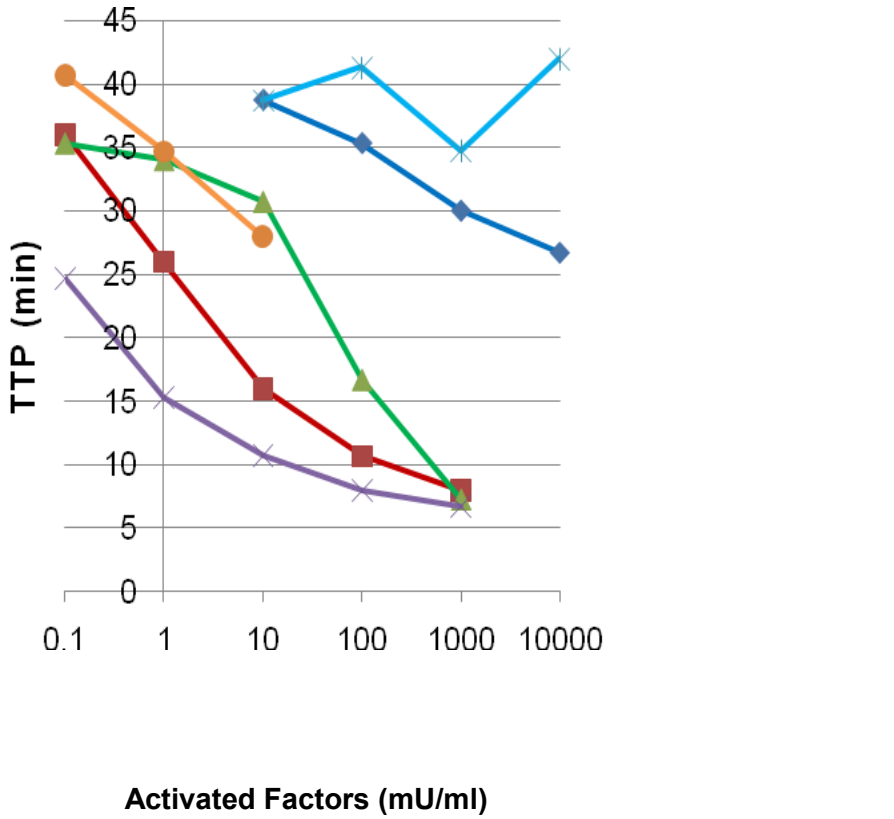
Illustration 14

Impact of activated factors on TGA/PTC in FXI-DP. An Octagam sample free from procoagulant potential, i.e. no activated factors were detectable, was spiked with commercially available activated and activity assigned proteases of the coagulation and contact phase systems.



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