Application Note

Quality Standards for Identity, Activity, and Purity Testing of Coagulation Factor VIIa

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Introduction

Eptacog alfa (activated) is a recombinant, human form of activated coagulation factor VII (rFVIIa) used for the treatment and prevention of bleeding episodes in patients with a range of conditions, such as hemophilia A and B, Factor II, V, VII, X, or XII deficiencies, or Von Willebrand's disease. rFVIIa is a 50 kDa, two-chain glycoprotein enzyme that results from the cleavage of a single peptide bond cleavage in FVII at Arg212[1]. The light chain is covalently linked to the heavy chain protease domain by a single disulfide bridge. The light chain anchors rFVIIa to the phospholipid cell surface and tissue factor while the heavy chain protease domain provides substrate binding specificity and catalytic activity[2].

Post translational modification of the recombinant protein is necessary for full bioactivity,[3] reinforcing the need for stringent quality control procedures that make use of validated reference materials.

rFVIIa Reference Standards for Identity, Biological Activity and Purity Testing

The USP Coagulation Factor VIIa Reference Standard (catalog # 1311500) and USP Coagulation Factor VIIa for Bioassay Reference Standard (catalog # 1311518) can be used as calibrators and comparators when testing samples of rVIIa products for identity, purity, and potency during manufacturing, storage, and throughout the supply chain.

- Coagulation Factor VIIa Reference Standard is supplied in a pure, lyophilized format.
- Coagulation Factor VIIa for Bioassay Reference Standard is provided in a formulation format to support bioassay testing without the need for excessive dilution, thus minimizing the risk of introducing experimental errors or variability.

Methods provided here or in the USP Certificates supplied with the USP Reference Standards are validated, allowing users to reduce or eliminate much of the labor and time required to develop new methods that require further evaluation and validation.

Identity Testing of rFVIIa Products

There are generally two ways used to identify a biologically produced material: chemical and biological. For chemical confirmation of identity, USP evaluated the sequence coverage, molecular mass, and digested fragments by peptide mapping. For biological confirmation of identity of rFVIIa products, USP used one-stage clotting method to confirm the bioidentity.

Sequence coverage to chemically confirm rFVIIa product identity

LC-MS method is used to chemically confirm the identity of rFVIIa products by analyzing amino acids sequence. USP Coagulation Factor VIIa Reference Standard was trypsin and chymotrypsin enzyme digested prior to analysis. and the resulting peptides were analyzed by LC-MS using the methods outlined in **Table 1**.

Sequence coverage maps of the chymotrypsin digest yielded 94.2% amino acid coverage of the heavy chain sequence and 42.1% amino acid coverage of the light chain sequence. Sequence coverage maps of the trypsin digest yielded 90.2% amino acid coverage of the heavy chain sequence and 54.6% amino acid coverage of the light chain sequence. Please refer to the USP Certificate for the USP Coagulation Factor VIIa Reference Standard to view the amino acid sequence. The results confirm the chemical identity of USP Coagulation Factor VIIa Reference Standard.

Table 1. LC-MS method parameters.

Mass spectrometer Conditions	
Ion Mode	ES positive
Mass Scan Range (m/z)	m/z 400-2000, MS/MS scan range varied
Sample	1.5 mg/mL, digested with 1 mg/mL trypsin

Chromatographic Conditions	
Column	Waters Acquity BEH 300 C18, 2.1 x 150 mm, 1.7 micron
Flow Rate (mL/min)	0.10
Injection Volume (µL)	50
Wavelength for PDA Detection (nm)	190-680
Fluorescence Detection Settings	Excitation 280 nm, emission 348 nm
Pressure (psi)	2750
Autosampler Temperature (°)	5
Column Temperature (°)	40
Mobile Phase A	0.1% Formic Acid in Water
Mobile Phase B	0.1% Formic Acid in Acetonitrile

Gradient Program		
Time (min)	**Flow Rate (mL/min)**	**Mobile Phase B %**
0	0.1	0
5	0.1	0
80	0.1	45
110	0.1	90
130	0.1	90
130.1	0.12	0
155	0.12	0

Molecular mass assessment to chemically confirm rFVIIa product identity

The molecular mass of the USP Coagulation Factor VIIa Reference Standard was determined using mass spectrometry by MALDI. A total of 1 µL of desalted sample was spotted to a MALDI plate along with 1 µL each of saturated sinapic acid (20 mg/mL) and 0.1% TFA for analysis. As shown in **Figure 1**, the molecular mass was determined to be 50865.411 Da, which agrees with the expected mass of ~50,000 Da.

Peptide mapping to chemically confirm rFVIIa product identity

The USP Coagulation Factor VIIa Reference Standard can also be used in peptide mapping as a comparator to determine the identity of rFVIIa products. For this purpose,

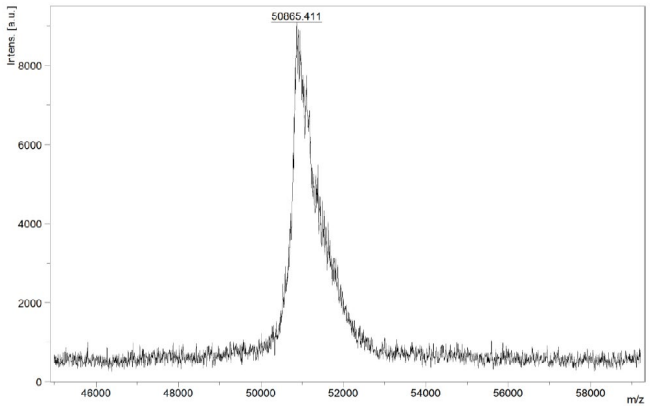


Figure 1. Figure 1. MALDI analysis of the USP Coagulation Factor VIIa Reference Standard.

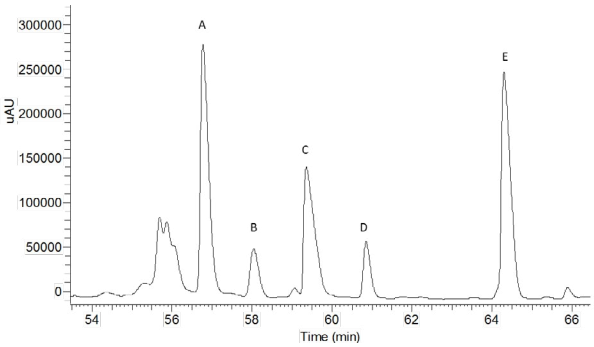


Figure 2. UV-HPLC of tryptic digest USP Coagulation Factor VIIa Reference Standard (catalog # 1311500).

the sample was digested into fragments without reduction or deglycosylation; fragments were separated using HPLC to create a distinctive "fingerprint" of five peaks (**Figure 2**). The amino acid sequences of these five peaks were confirmed by mass spectrometry.

Biological Identity Testing of rFVIIa Products

Biological activity of Coagulation Factor VIIa is commonly assessed using a potency assay (one-stage clotting method) that measures the ability of the protein to reduce the prolonged coagulation time of factor VII-deficient plasma. The one-stage method is based on the activated partial thromboplastin time (APTT or aPTT) clotting method. The method measures the ability of a sample containing the coagulation factor of interest to shorten the clotting time of Factor VII deficient plasma. The amount of time reduced

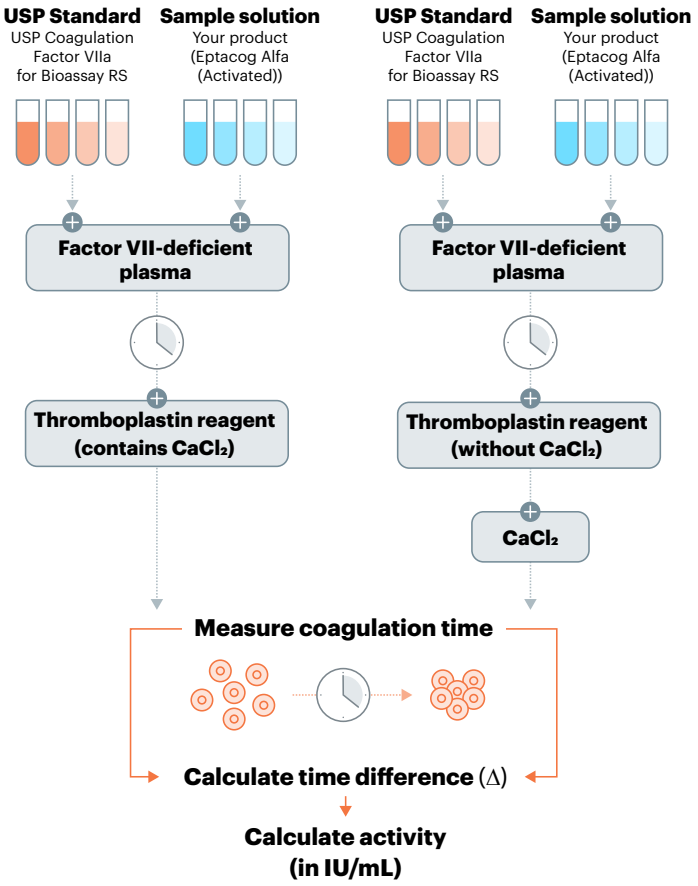


Figure 3. Experimental Procedures for Eptacog Alfa (Activated) Bioidentity Assay using the One-Stage Clotting Method

varies with the experimental system; therefore, there is not a specific requirement.

The method relies on the sequential and linked macromolecule interactions that are part of the intrinsic and common pathways of coagulation. The method requires factor deficient plasma, a source of phospholipids containing an added contact activator, the sample containing the clotting factor to be measured by the method, and calcium chloride. The assay response is the formation of a plasma gel-like clot which can be detected by mechanical or photo-optical means. The time required for clot formation, the clotting time, is considered directly dependent on the clotting factor activity present in the sample being tested. The experimental procedure for this assay in the certificate accompanying the Coagulation Factor VIIa for Bioassay Reference Standard, illustrated in **Figure 3**, can be found in the "Appendix".

The USP Coagulation Factor VIIa for Bioassay Reference Standard content per ampule was determined by testing against the World Health Organization's (WHO) 2nd International Standard for rVIIa Concentrate (NIBSC Code 07/228, lyophilized). Candidate material was provided to external collaborator laboratories as blinded duplicate samples (Sample A and Sample B). Each lab performed three independent assays on three different days for both samples. Results obtained for Sample A and Sample B were compared by statistical analysis and no significant difference in potency was observed (**Table 2**). Results for both sample A and B were combined to establish the potency value of the candidate lot as 577 IU per ampule with a 95% CI of 560 – 595 IU per ampule (**Table 2**).

Table 2. Results of bioidentity assay performed on two samples of the candidate Coagulation Factor VIIa for Bioassay Reference Standard. Note: Lab 1 ran six assays on six different days, instead of three assays on three different days. The results (IU/ampule) obtained on the additional days were 551, NR, and 573 for Sample A and 588, 577, 566 for Sample B. NR = not reported

Potency Value of the Candidate Lot, (IU/ampule)							
Sample	**Day**	**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**	**Lab 6**
A	1	522	566	NR	571	579	NR
	2	557	594	560	582	552	665
	3	584	565	609	578	534	550
B	1	489	594	NR	550	588	532
	2	602	554	651	580	569	NR
	3	574	598	593	546	551	612
Mean		562	579	603	568	562	590
%RSD		5.7	3.3	6.3	2.8	3.6	10.3
Overall Mean		577					

Determination of rFVIIa Product Concentration

To determine the concentration of rFVIIa, a validated HPLC method is described in **Table 3**. The same method is used to evaluate the presence of dimers and related substances of higher molecular mass. **Figure 4** shows the chromatograms generated using this method to analyze the USP Coagulation Factor VIIa Reference Standard.

Impurities Testing of rFVIIa Products: Degraded Heavy Chain and Oxidized Forms

The validated HPLC method outlined in **Table 4** can be used to determine the presence of degraded heavy chain and oxidized forms of rFVIIa in test samples. These impurities result from the manufacturing process. Most impurities will be removed through the downstream purification process, but removal may not be 100% effective. Therefore, testing

Table 3. HPLC parameters for determination of Factor VIIa concentration.

Chromatographic Conditions	
Column	Waters Protein-Pak 300SW (BBL-55), 30 cm x 7.5 mm, 10 µm packing with L59
Sample Concentration mg/ml	1.5
Flow Rate (mL/min)	0.5
Injection Volume (µL)	20
Wavelength (nm)	215
Fluorescence Detection Settings	Excitation 280n nm, emission 348 nm
Pressure (psi)	2750
Column Temperature (°)	25
Run Time (minutes)	30
Mobile Phase	0.2 M ammonium sulfate with 5% 2-propanol
Solvent	Water

Table 4. HPLC parameters for separation of degraded heavy chain and oxidized forms of rFVIIa.

Chromatographic Conditions	
Column	Pharma-tech C4 column 25 cm x 4.0 mm, 5µm
Sample Concentration mg/ml	1.5
Flow Rate (mL/min)	1.0
Injection Volume (µL)	20
Wavelength (nm)	214
Column Temperature (°)	65
Run Time (minutes)	50
Mobile Phase	Solution A: 0.1% Trifluoroacetic acid Solution B: Acetonitrle, water and trifluoracetic acid (800:200:0.9)
Solvent	MilliQ H_2O

Gradient Program		
Time (min)	**Solution A (%)**	**Solution B (%)**
0	56.5	43.5
30	43.5	56.5
33	43.5	56.5
33.1	0	100
38	0	100
40	56.5	43.5
50	56.5	43.5

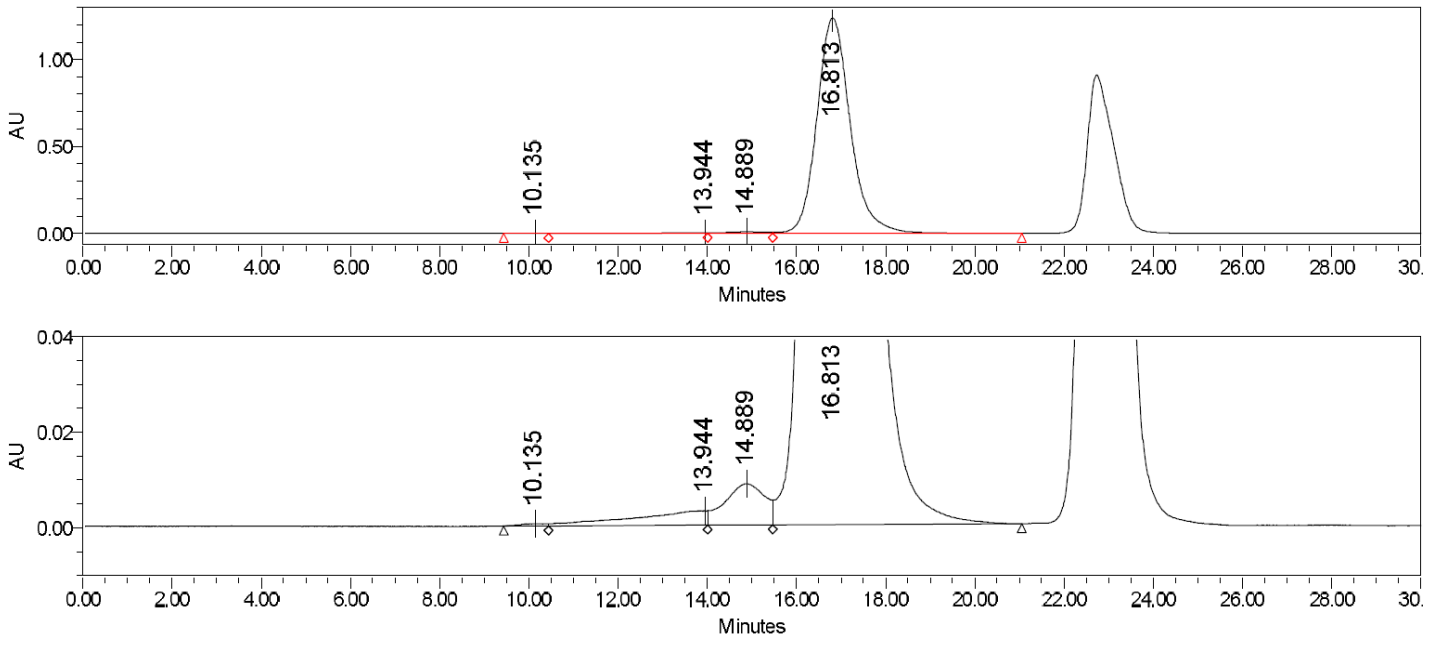
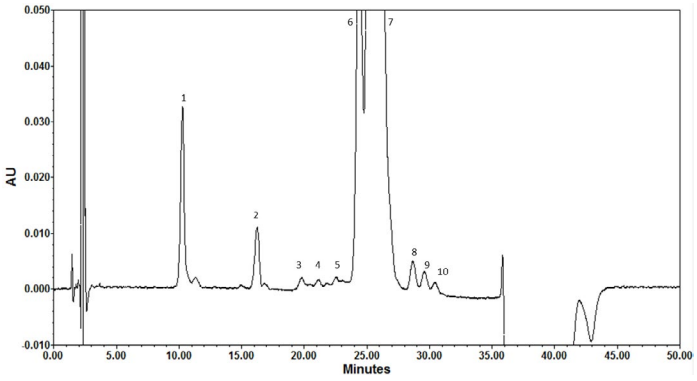


Figure 4. HPLC chromatogram of USP Coagulation Factor VIIa Reference Standard for the determination of Factor VIIa concentration (bottom figure is a zoomed in version of the top figure, allowing for clear visualization of the impurity peaks).



Peak	Name	Typical Retention Time (min)
1	Degraded heavy chain form, amino acids 1–290	10
2	Degraded heavy chain form, amino acids 291–406	16
3–5	Oxidized forms, methionyl sulfoxide of rFVIIa at Met^{298}, Met^{306}, and Met^{327}, respectively	19–24
6	Degraded heavy chain form, cleaved between Arg^{315} and Lys^{316}	25
7	rFVIIa	26
8–10	Unknown	29–32

Figure 5. Separation of degraded heavy chain and oxidized forms of USP Coagulation Factor VIIa Reference Standard.

is performed to determine the level of these impurities to control product quality. Separation of degraded heavy chain and oxidized forms of USP Coagulation Factor VIIa Reference Standard is shown in **Figure 5**.

Impurities Testing of rFVIIa Products: Gla-Domainless Forms

USP provides an HPLC method for determining the presence of the Gla-domainless (γ-Carboxylation) form of coagulation factor VIIa in test samples (**Table 5**). Separation of the Gla-domainless form of USP Coagulation Factor VIIa Reference Standard is shown in **Figure 6**.

Table 5. HPLC parameters for separation of Gla-domainless forms (γ-Carboxylation) of rFVIIa.

Chromatographic Conditions	
Column	Waters 25 cm x 4.0 mm, 13 µm
Sample Concentration mg/ml	1.5
Flow Rate (mL/min)	1.0
Injection Volume (µL)	100
Wavelength (nm)	280
Column Temperature (°)	25
Run Time (minutes)	35
Mobile Phase	Solution A: 10 mM Tris Base + 10 mM bis-tris propane, pH 9.4 Solution B: 10 mM Tris Base + 10 mM bis-tris propane + 1.4 M ammonium acetate, pH 9.4
Solvent	MilliQ H_2O

Gradient Program		
Time (min)	**Solution A (%)**	**Solution B (%)**
0	100	0
2.5	100	0
27.5	0	100
30.5	100	0

Glycosylation Pattern Analysis of rFVIIa Products

As noted above, rFVIIa carries complex post translational modifications (PTMs) that influence its pharmacokinetics and pharmacodynamics. These PTMs include γ-carboxylation,

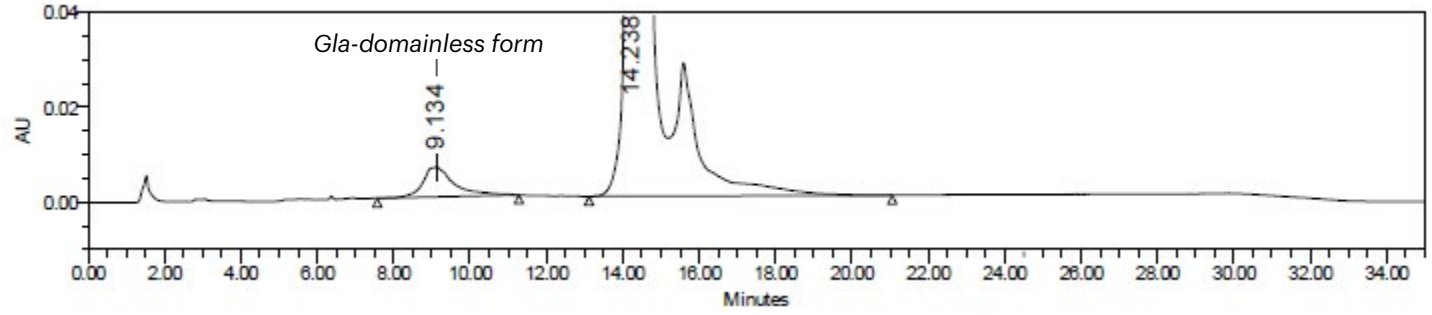
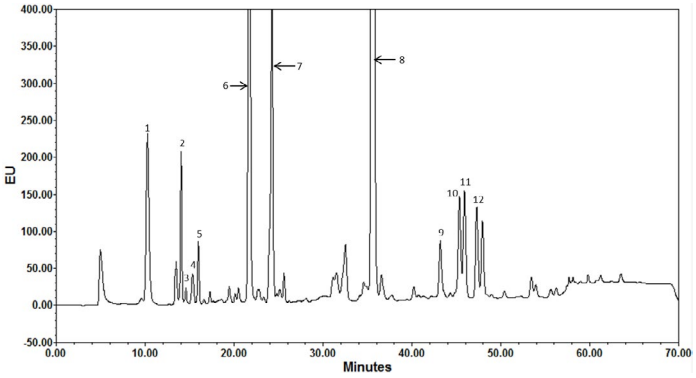


Figure 6. Separation of Gla-domainless forms (γ-Carboxylation) of USP Coagulation Factor VIIa Reference Standard.



Peak	Charged	Structure	Typical Retention Time (min)
1	No	Core fucosylated biantennary – non sialylated (two *N*-acetylgalactosamine terminals)	12
2	No	Core fucosylated biantennary – non sialylated (*N*-acetylgalactosamine and galactose terminals)	15
3	No	Structure not determined	16
4	No	Core fucosylated biantennary – non sialylated (galactose and *N*-acetylglucosamine terminals)	17
5	No	Core fucosylated biantennary – non sialylated (two galactose terminals)	17.5
6	Yes	Core fucosylated biantennary – monosialylated (and one *N*-acetylgalactosamine terminal)	23
7	Yes	Core fucosylated biantennary – monosialylated (and one galactose terminal)	26
8	Yes	Core fucosylated biantennary – bisialylated	38
9	Yes	High-mannose structure with one phosphate group	44
10, 11	Yes	Core fucosylated triantennary – trisialylated	47, 48
12	Yes	Structure not determined	49

Figure 7. Representative glycan profile for the USP Coagulation Factor VIIa Reference Standard and identification of peaks.

N- and *O*-linked glycosylation, and β-hydroxylation and can vary depending on the cell lines used to produce the recombinant protein.

USP provides an analytical method suitable for determining the glycosylation pattern of rFVIIa (please refer to USP <212> *Oligosaccharide Analysis* for method details). **Figure 7** shows an example of an *N*-glycan analysis of the USP Coagulation Factor VIIa Reference Standard showing the twelve expected glycan peaks and their identification.

Conclusion

Ensuring the identity, quality, and biological activity of rVIIa is essential to deliver the desired therapeutic effect and ensure patient safety.

The USP Coagulation Factor VIIa Reference Standards and USP Coagulation Factor VIIa for Bioassay Reference Standard serve as comparators for the chemical integrity and biological activity of rVIIa and can be used to support analytical method development, validation, release testing, stability testing, and analytical method transfer. When manufacturing rFVIIa, the USP Reference Standards, combined with validated methods, enable easy analytical testing to demonstrate acceptable product quality without the risks associated with developing new methods and in-house standards.

USP routinely tests all Reference Standards and maintains a Continued Suitability of Use (CSU) study to evaluate material stability, such as ensuring assigned potency values do not shift or drift over time. When a new reference standard lot is released, tests are performed to bridge with the current lot to minimize lot-to-lot variation, thus maintaining high quality standards.

References

1. Giansily-Blaizot M and Schved J-F. Recombinant Human Factor VIIa (rFVIIa) in Hemophilia: Mode of Action and Evidence to Date. Therapeutic Advancements in Hematology. 2017; 8(12):345-352. doi: 10.1177/2040620717737701

2. Shobe J, Dickinson CD, Edgington TS, et al. Macromolecular substrate affinity for the tissue factor-factor VIIa complex is independent of scissile bond docking. J Biol Chem 1999; 274: 24171–24175. doi: 10.1074/jbc.274.34.24171

3. Chevreux G, Tilly N, Leblanc Y, Ramon C, Faid V, Martin M, Dhainaut F, Bihoreau N. Biochemical characterization of LR769, a new recombinant factor VIIa bypassing agent produced in the milk of transgenic rabbits. Haemophilia. 2017; 23:e324–e334, doi: 10.1111/hae.13253

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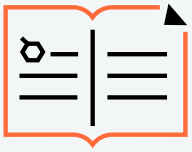
USP has developed several general chapters containing best practices to help develop and validate bioassays.



- <111> *Design and Analysis of Biological Assays*
- <1030> *Biological Assay Chapters – Overview and Glossary*
- <1032> *Design and Development of Biological Assays*
- <1033> *Biological Assay Validation*
- <1034> *Analysis of Biological Assays*
- <1108> *Assays to Evaluate Fragment Crystallizable (FC)—Mediated Effector Function*

Appendix

Experimental Procedures for Eptacog Alfa (Activated)
Potency Assay:

Potency is assessed by comparing the dose-response curve of the Sample solution to that of the Standard solution. Use a suitable coagulation analyzer or carry out the assay with incubation tubes and reagents maintained at 37°.

Diluent: Use a suitable buffer for the coagulation test. [NOTE – The buffer should contain a protein carrier such as bovine or human albumin that will limit the adsorption of the sample and Standard dilutions onto the surface of the test tubes. An example of a suitable buffer is 0.05 M piperazine-1,4-bis(2-ethanesulfonic acid), 0.1 M sodium chloride, 2 mM sodium edetate, and 1% bovine albumin, adjusted to a pH of 7.2].

Factor VII-deficient plasma: Use a suitable Factor VII-deficient plasma.

Thromboplastin reagent: Use a suitable thromboplastin reagent.

Calcium chloride solution: Use a calcium chloride solution if the thromboplastin reagent used does not contain calcium chloride.

Standard solution: Prepare solutions of USP Coagulation Factor VIIa for Bioassay RS in Diluent for at least four different concentrations within the linear range. Prepare in duplicate and use the solutions immediately. [Note- An example of the linear range is 0.005-0.14 IU/mL.]

Sample solution: Prepare solutions of Eptacog Alfa (Activated) in Diluent for at least three and preferably four different concentrations within the linear range of the assay. Prepare in duplicate and use the solutions immediately. [Note- An example of the linear range is 0.005–0.14 IU/mL. The range is dependent on the factor VII-deficient plasma and thromboplastin reagent used.]

Analysis

Samples: Standard solution and Sample solution

Test Procedures:

To 40 µL each of Standard solution or Sample solution, add 40 µL of factor VII-deficient plasma, incubate for an appropriate time at 37°, and add 80 µL of Thromboplastin reagent.

For thromboplastin reagents that contain calcium chloride, measure the coagulation time (the interval between the addition of Thromboplastin reagent and the formation of a fibrin clot). For thromboplastin reagents that do not contain calcium chloride, add an appropriate amount of calcium chloride (after the addition of Thromboplastin reagent) and measure the coagulation time from the addition of calcium chloride to the formation of a fibrin clot.

Calculate the activity, in IU/mL, using an appropriate statistical method and validity test. For example, the parallel-line assay and the statistical tests for linearity, slope, and parallelism for the sample compared to the standard must pass at the 95% level. The confidence limits (P = 0.95) must be within 80%–125% of the estimated potency.

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