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# Two-Step Purification of Coagulation Factor IX (FIX) from Cryopoor Plasma Using CIMmultus® DEAE and SO4 Columns

Mojca Zafran Lulik[1], Jana Vidič[1], Marija Begić Kokan[1], Neža Črnigoj[1], Jakob Murovec[1], Ažbe Žnidaršič[1]*, Lucija Dončević [2], Tomislav Friganović[2]

[1] Sartorius BIA Separations d.o.o., Ajdovščina, Slovenia
[2]Genos Glycoscience Research Laboratory, Zagreb, Croatia
*Corresponding author: azbe.znidarsic@sartorius.com

## Introduction

Coagulation Factor IX (FIX) is a vitamin K-dependent protein that plays a crucial role in the blood clotting process, essential for stopping bleeding. A deficiency in Factor IX leads to Haemophilia B, a genetic disorder characterized by excessive bleeding due to the inability to form proper blood clots. Conventional plasma-derived FIX manufacturing using diffusive based resins is lengthy and often has limited efficiency in removing co-enriched vitamin K-dependent coagulation factors.

To address these constraints, a two-step CIM Convective Interaction Media®monolithic chromatography process was developed to streamline downstream purification by enabling rapid and direct loading of large volumes of undiluted cryopoor plasma and delivering highly pure FIX.

## 1. Experimental approach

50 CV of undiluted cryopoor plasma was loaded directly to CIMmultus DEAE monolithic column with 6 µm channels (Sartorius BIA Separations, Slovenia) at 3 CV/min at RT, followed by 5x dilution of DEAE eluate and loading to CIMmultus SO4 monolithic column with 2 µm channels (Sartorius BIA Separations) at 2.5 CV/min (Figure 1).

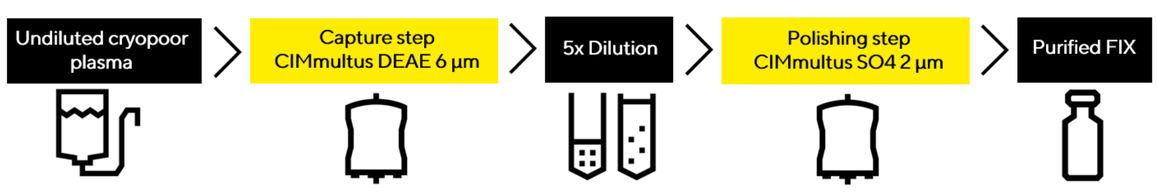



Figure 1: Schematic diagram of the Coagulation Factor IX purification process. The scheme does not include a viral inactivation step (recommended between the two chromatography steps) nor the final product formulation step.

FIX in the samples was quantified using the Rox Factor IX (Rossix, Sweden) chromogenic kit with a limit of detection of about 0.001 IU/mL FIX. To determine the purity of the samples, SDS-PAGE analysis was performed (gel and staining kit from Invitrogen, USA). Concentration of other coagulation factors (e.g. FII, FVII and FX) was measured with STA Compact Max coagulometer (Stago,France). Specific activity of FIX was determined using two methods; the Bradford protein assay and spectrophotometrically with a NanoDrop spectrophotometric instrument (Thermo Fisher Scientific, USA).

## 2. High elution selectivity with CIMmultus SO4

A polishing chromatography step was added to further purify FIX from other proteins, including other Vitamin K–dependent coagulation factors (FII, FVII, FX). Figure 2 shows the chromatogram of chromatographic polishing step using CIMmultus SO4 column, after loading the elution with the highest FIX concentration from the first chromatographic step using CIMmultus DEAE column. UV280 trace indicates that majority of protein-rich species was present in the flowthrough or eluted during CIP phase. The main elution peak, marked black square, indicate the fraction with the highest amount of FIX, whose presence was confirmed using the analytical method Rox Factor IX.

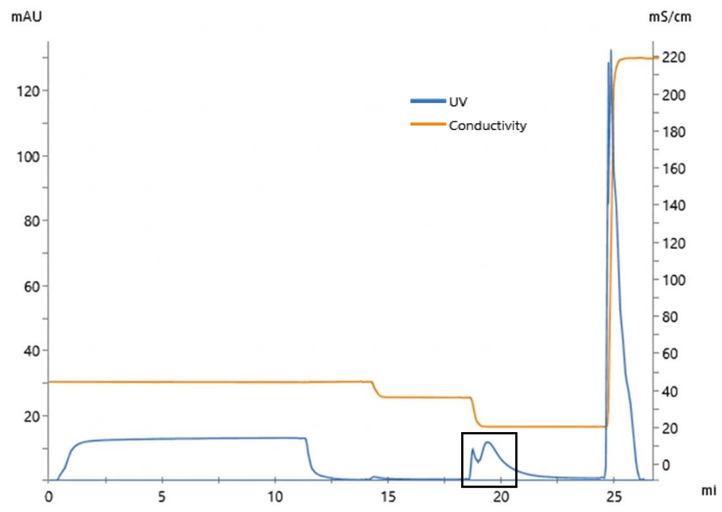



Figure 2: Chromatogram after the second chromatographic polishing step using CIMmultus SO4 column. The solid blue line represents the UV signal at 280 nm, while the orange line represents conductivity. The main elution peak with the highest amount of FIX is marked with a black square.

The column was equilibrated using a mildly acidic buffer with moderate salt concentration. Elution was performed in two steps, each at a higher pH and lower salt concentration than the previous one.

## 3. Results - recovery of FIX in both chromatographic steps and specific activity

Table 1 shows the concentrations, biological activity values and recovery results of FIX in chromatographic fractions: FIX recovery of elution in CIMmultus DEAE step was 49%, while the recovery of the CIMmultus SO4 step was 93%.
The specific activity of FIX for the main elution after CIMmultus SO4 step is 455 IU/mg of protein.

| Step 1 \| CIMmultus DEAE monolith | | | | Step 2 \| CIMmultus SO4 monolith | | | |
|---|---|---|---|---|---|---|---|
| Fraction | FIX (IU/mL) | FIX (IU) | Recovery (%) | Fraction | FIX (IU/mL) | FIX (IU) | Recovery (%) |
| Load | 1.0 | 47.8 | / | Load – DEAE Elution 1.2 | 1.8 | 19.5 | / |
| FT fraction | 0.0 | 1.7 | 4 | FT fraction | 0.1 | 1.2 | 6 |
| Elution 1.1 | 1.2 | 3.6 | 7 | Main elution | 4.6 | 18.2 | 93 |
| Elution 1.2 | 6.3 | 19.0 | 40 | / | / | / | / |
| Elution 1.3 | 0.3 | 0.9 | 2 | / | / | / | / |
| Total | | 25.2 | 53 | Total | | 19.8 | 99 |

Table 1: Concentration, biological activity values and recovery results of FIX in chromatographic fractions from both purification steps

## 4. Results - removal of vitamin-K dependent factors

One of the main purification challenges in FIX purification is the removal of vitamin K-dependent serine proteases - Prothrombin (FII), FVII and FX, frequently chromatographically overlapping with FIX due to similar biophysical characteristics (charge, size etc.). Figure 3 shows biological activity values in main chromatographic fractions for both purification steps. In the main elution of the CIMmultus SO4 purification step, FII, FVII and FX are below the limit of quantification.

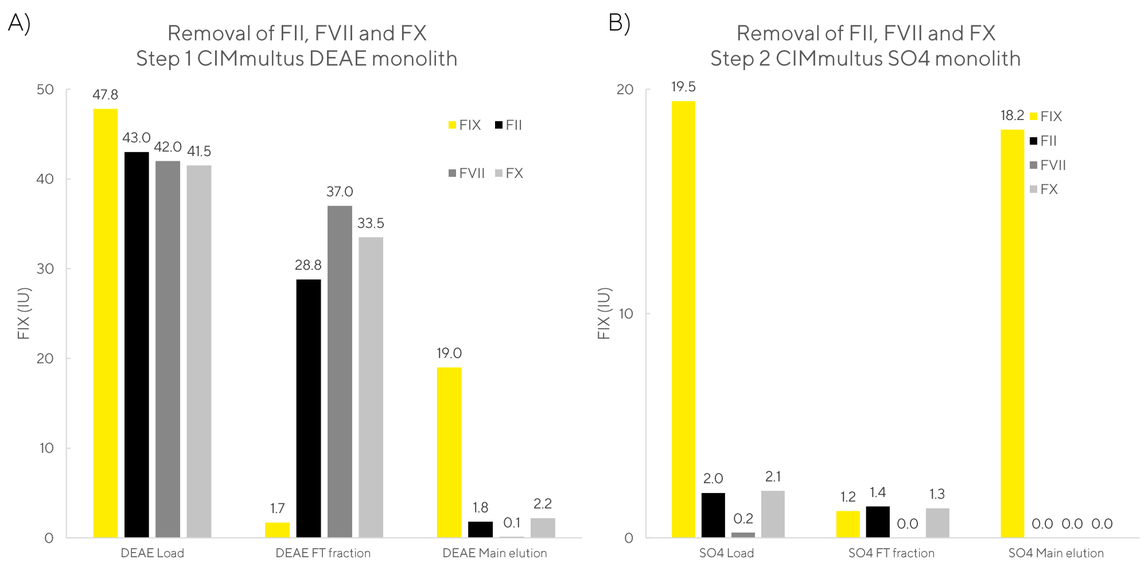



Figure 3: Removal of impurities (FII, FVII, FX) during the CIMmultus DEAE (A) and CIMmultus DEAE+SO4 steps (B).

## 5. Results – purity of chromatographic fractions using SDS-PAGE analysis and Western Blot for Vitronectin

Purity of FIX against all impurities of the main chromatographic fractions was visually assessed with SDS-PAGE. The last lane on Figure 4A represents elution after second chromatographic polishing step using CIMmultus SO4 column, proving the high purity of FIX. FIX is marked with a yellow square.

Besides coagulation factors, proteolytic fragments of vitronectin is visualized at similar size on SDS-PAGE gel as FIX. Using Western Blot analysis, we determined that vitronectin (approx. 65 - 75 kDa) was present in the main elution after the CIMmultus DEAE chromatographic step, while there was no observable presence of vitronectin in the main elution of the CIMmultus SO4 chromatographic step. Since no vitronectin band was detected in the flow-through (FT) fraction, it was most likely eluted during the CIP step (Figure 4B).

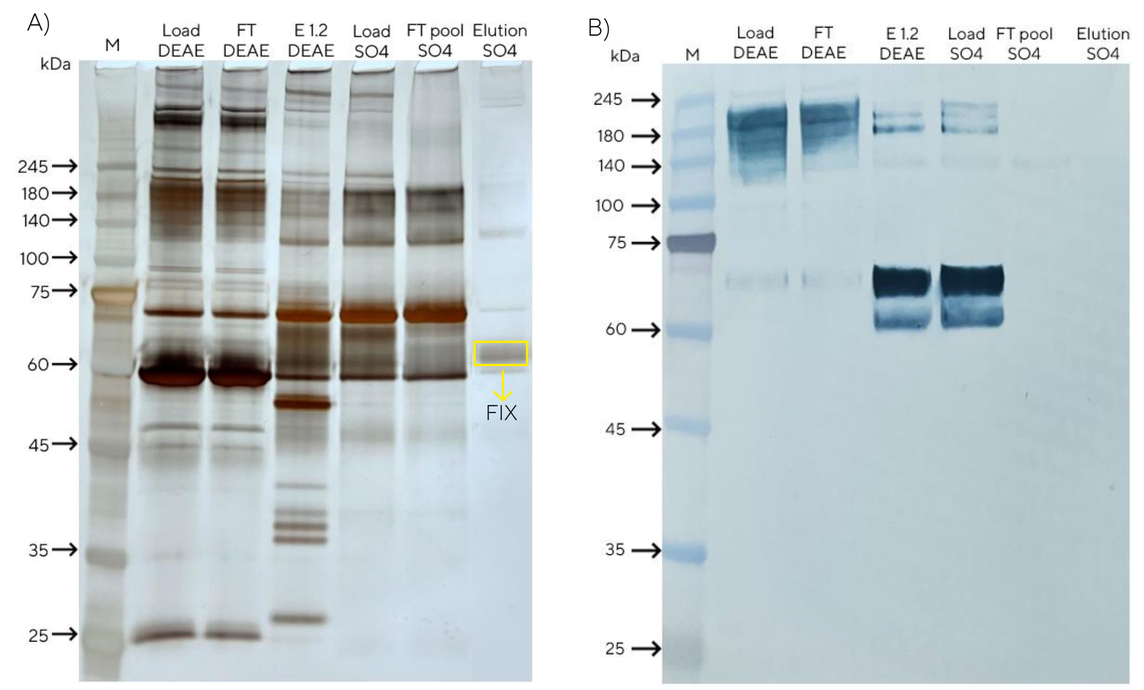



Figure 4: SDS-PAGE (nonreduced) analysis (A) and Western blot for vitronectin (B) of chromatography fractions from both purification steps shows high purity of SO4 main elution.

## 6. Conclusion

The study revealed that CIMmultus DEAE and SO4 columns are effective in selectively binding and purifying FIX from cryopoor plasma. The data presented demonstrate that the use of both monolithic columns allows:

- Rapid and direct loading of large volumes of undiluted cryopoor plasma
- High process recovery, with almost full recovery of the polishing step using CIMmultus SO4.
- The efficient removal of coagulation factors FII, FVII, and FX, which are reduced to levels below the limit of detection.
- High specific activity (455 IU/mg of protein) and purity, confirmed by SDS-PAGE analysis
- The effective removal of vitronectin, a well-known impurity in FIX purification, with no observable presence after the purification process.

## 7. Literature

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Branović, K., Buchacher, A., Barut, M., Štrancar, A., & Josić, D. 2000. Application of monoliths for downstream processing of clotting factor IX. Journal of Chromatography A, 903(1–2), 21–32

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