For use in quality control / manufacturing process only.

FcRn Affinity Column Gen2

Version: 01
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Prepacked column with immobilized human FcRn receptor for affinity chromatography.

Cat. No. 09 430 857 001 1 column

Store the column at +2 to +8°C.

1. General Information

1.1. Contents

Label	Function / Description	Content
FcRn Affinity Column Gen2	▪ Prepacked chromatography column with 1 mL bed (matrix) volume of FcRn-Biotin-Streptavidin-Sepharose resin. ▪ Preassembled with the optimal fittings and filters applicable on standard liquid chromatography systems, such as FPLC or HPLC. ▪ The resin is supplied in storage buffer: 16 mM MES-Na, 4 mM Tris-HCl, 140 mM NaCl, 20 tablets/liter cOmplete, 0.05% Kathon CG, pH 6.0. ▪ Ready-to-use and reusable. *There may be a light white line in the middle of the column. This is not a weakness of the material. The mark is commonly generated in the manufacturing process and does not influence the stability.*	1 column

1.2. Storage and Stability

Storage Conditions (Product)

When stored at +2 to +8°C and prior to using, the column is stable through the expiry date printed on the label.

Label	Storage
FcRn Affinity Column Gen2	Store at +2 to +8°C.

Stability

After first use, the stability is no longer limited by the expiry date, it depends on the regular treatment with fresh buffer, the protection against microbial contamination, and the intensity of usage. Under typical conditions, the function is stable for at least 100 runs. Thereafter, check the separating capacity with an appropriate control standard, we recommend the FcRn IgG Control*.

Label	Stability
FcRn Affinity Column Gen2	After usage of the column, for short-term storage, use 80% Buffer A and 20% Buffer B. For long-term storage periods >4 weeks, use the Storage Buffer for protection. *See buffer compositions in section* **Working Solution**.

1.3. Additional Equipment and Reagent required

Instruments and consumables

- Polypropylene tubes for preparation of sample dilutions
- Micropipettes
- Glass bottles for preparation of buffers
- 0.2 µm membrane filter
- pH meter
- Syringes for sample injection
- Liquid Chromatography System equipped with UV/Vis detector and data analysis software. Use of in-line vacuum degasser, auto-sampler, column oven, and pH measuring cell are recommended.

Reagents

See section, **Working Solution** *for additional information on preparing solutions.*

- MES sodium salt
- Tris base
- HCl
- NaOH
- NaCl
- Double-distilled water
- Positive control: FcRn IgG Control*
- For the long-term storage buffer:
  - cOmplete Protease Inhibitor Cocktail, 20 tablets (MilliporeSigma/Merck KGaA 04693116001)
  - ProClin-300 (MilliporeSigma/Merck KGaA 48912-U)

1.4 Application

The column is designed for chromatographic differentiation of structural variants of monoclonal IgG depending on their different binding affinity to the neonatal Fc receptor (FcRn). FcRn analysis can provide insights into the structural and functional integrity of therapeutic immunoglobulins and enables improved conclusions on the half-life of the immunoglobulins *in vivo*.

The recombinant FcRn immobilized in this chromatography column has a specific binding affinity to IgG molecules. The strength of the binding depends on structural variations of the IgG, like different Fab fragments, mutations in the Fc part, antibody isotypes, oxidized forms, aggregates, immune complexes, or antibody-drug conjugates. The FcRn affinity chromatography enables differentiation of such variants by peak patterns and retention times in the elution profile.
Furthermore, the FcRn receptor has a binding affinity also to serum albumin, and the FcRn affinity chromatography can also be used to characterize albumin preparations, for example, for differentiation of oxidized, aggregated, or conjugated variants.

Proteins binding to FcRn:

- Human IgG1, IgG2, IgG3, IgG4
- Some IgG of non-human species are able to bind human FcRn
- Human albumin (often used as a fusion protein for therapeutic proteins)

2. How to Use this Product

2.1. Before you Begin

Sample Materials

The FcRn Affinity Column Gen2* is designed for analytics of purified preparations of:
- IgG
- Albumin

Control Reactions

Positive control

Use the FcRn IgG Control* to verify the correct separation performance of the column.
Alternatively, a suitable preparation of monoclonal antibodies consisting of at least two distinguishable variants can be used, or, bovine serum albumin (BSA), such as MilliporeSigma/Merck KGaA A2153. BSA must be of ≥96% purity and purified by cold ethanol fractionation.

⚠ ***Do not use liquid BSA formulations; always prepare a fresh solution from a lyophilized BSA preparation, dissolving 5 mg BSA in 1 mL of Buffer A.***

Negative control

Use Buffer A as a negative control.

Working Solution

Preparation of the required buffer solutions

⚠ ***The binding affinity may drop significantly if the buffer composition is suboptimal.***

ⓘ *The functionality of the FcRn Affinity Column Gen2 has been tested using the recommended Buffer A and Buffer B specified in the table below. Other buffers may function as well, but the performance needs to be evaluated individually.*

Component	Composition
Buffer A	20 mM MES-HCl, pH 5.5, 140 mM NaCl
Buffer B	20 mM Tris-HCl, pH 8.8, 140 mM NaCl
Storage Buffer (long-term)	80% Buffer A and 20% Buffer B, pH 6.0, with addition of cOmplete Protease Inhibitor Cocktail (1 tablet/50 mL) and ProClin-300 (0.05%).

2.2. Protocols

Handling instructions for the column

The resin in the FcRn Affinity Column Gen2 must always be maintained in buffer and never be allowed to dry. Use the buffers recommended by Roche and do not apply ethanol or other organic solvents to the column.

Technical specifications

Matrix	Streptavidin Sepharose
Bead size	34 µm
Column (resin bed) volume	1 mL
Binding capacity	≥100 µg IgG per mL bed volume of resin.
Maximal system pressure and temperature	20 bar at +25°C.
Recommended maximal volumetric flow rate	0.5 mL/min
Reusability	Columns can be reused for at least 100 injections.
Recommended buffer for long-term storage >4 weeks	80% Buffer A and 20% Buffer B, pH 6.0, with addition of cOmplete Protease Inhibitor Cocktail* (1 tablet/50 mL) and ProClin-300 (0.05%) for stabilization.
Recommended running buffer during chromatography	80 to 0% Buffer A, 20 to 100% Buffer B, pH 5.5 to 8.8.

Preparation of sample material

Dilute samples with Buffer A to a protein concentration of approximately 1 mg/mL.

i *Sample and positive control solutions must be free of particles. Solutions can be treated by centrifugation or filtered with a non-absorbing membrane, such as PES.*

i *When injecting 30 µL of sample, a minimum volume of 40 µL is required.*

System preparation

FcRn Affinity Columns Gen2 and the described protocol are compatible with common automated chromatography systems, such as the Agilent Series 1100 HPLC system.

1. Set UV/Vis detector wavelength to 280 nm.

2. Cool auto-sampler, if available, to +2 to +8°C.

3. Filter Buffers A and B through a 0.2 µm membrane filter before use. The Buffers can be used at +15 to +25°C.

4. Temper column oven, if available, to +25°C. Alternatively, use the column at +15 to +25°C.

Protocol

1. Wash the pump with Buffer B, followed by Buffer A, using the System Wash function of the Liquid Chromatography System, according to the recommendations by the manufacturer. Make sure all air is displaced from the pumps and tubings of the system.

2. Ensure correct flow direction, shown with the arrow imprinted on the column. Remove the plug at the column outlet and attach it to the outlet tubing of the chromatography system.

   i *Save the plug of the column outlet for recapping after use.*

   i *Do not remove the beige PEEK column guard. Instead, use it for connection of the outlet tubing.*

3. As soon as Buffer A is running out of the inlet tubing of the chromatography system, remove the upper plug from the column and immediately attach it to the inlet tubing of the chromatography system with a drop-to-drop connection. Continuously measure OD_{280} values on the chromatography system.

Save the plug of the column inlet in case the column needs to be stored or is to be reused.
Do not remove the beige PEEK column guard. Instead, use it for connection of the inlet tubing.

4. Define the flow rate as 0.5 mL/min and equilibrate the column with 5 column volumes of Buffer A or until a stable OD_{280} baseline is reached.

5. Pause the run. Load 30 µL of sample or control onto the column with a volumetric flow rate of 0.5 mL/min.

6. Elute the protein according to the gradient in the following table:

Time [min]	% Buffer A	% Buffer B
0	80	20
10	80	20
80	0	100
90	0	100
93	80	20
103	80	20

7. Repeat Steps 5 and 6 two times to ensure that the interactions between sample/control and column material have reached equilibrium and elution profiles are reproducible.

8. Analyze data by recording, for example, peak elution, area percent, pH, or buffer gradient, etc. The first run of each individual sample/control is expected to differ in peak area distribution compared to subsequent runs. Roche recommends excluding the first run from data analysis.

9. Equilibrate for the next run,
– or store column at +2 to +8°C in 80% Buffer A, 20% Buffer B, pH 6.0,
– or rinse column with Storage Buffer for long-term storage >4 weeks.

The first run of each individual sample/control can be shortened by making the pH gradient steeper. For example, use the program: 0 minute - 20% B, 10 minutes - 20% B, 15 minutes - 100% B, 25 minutes - 100% B, 28 minutes - 20% B, 38 minutes - 20% B.

Checks and controls

FcRn Affinity Columns Gen2 can be used multiple times without loss of binding or separation capacity. Over time, however, protein aggregates might accumulate, leading to a decrease in the performance of the resin within the columns. This can be identified by a slower flow rate or a higher back pressure. The performance of the column can be evaluated by a series of controls:

Positive control

Use the FcRn IgG Control*. Alternatively, inject a suitable preparation of antibody variants, or a freshly prepared BSA solution and run the gradient according to Step 6. Elution peaks should occur in the range of 10 to 80 minutes. Peaks of variants, such as monomers/dimers, should be clearly distinguishable (peak-valley-peak).

Negative control

Inject Buffer A and run the gradient according to Step 6. No elution peak should occur in the range of 10 to 80 minutes.

3. Results

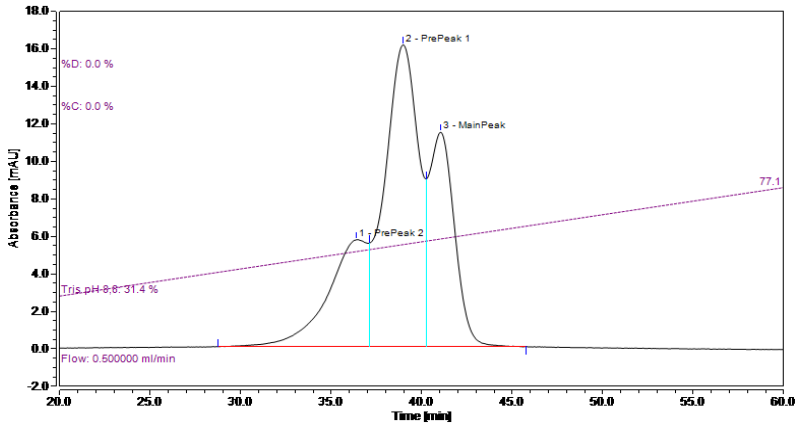

Fig. 1: Elution profile of the FcRn IgG Control*. Non-oxidized (MainPeak), partially oxidized (PrePeak 1), or completely oxidized (PrePeak2) methionine species can be differentiated. Retention times and peak area distribution may vary depending on capillary length, UV-cell volume and other individual system attributes.

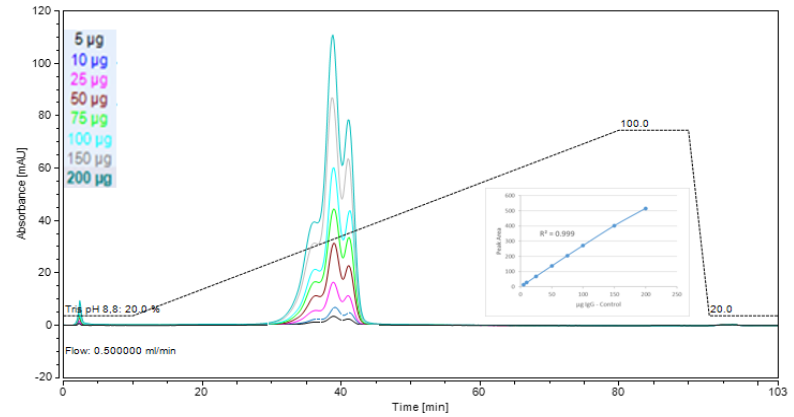

Fig. 2: Overlay of the elution profiles of consecutive runs with increasing amounts of the FcRn IgG Control*. The resulting integrated peak areas show a linear dependence on the injected amount of the IgG in the tested range of 5 to 200 µg IgG.

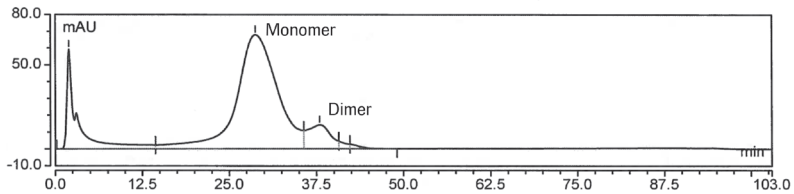

Fig. 3: Elution profile of a BSA solution. Monomer and dimer species can be differentiated. Retention times and peak area distribution may vary depending on capillary length, UV-cell volume, and other individual system attributes.

4. Troubleshooting

Observation	Possible cause	Recommendation
Air bubbles in the resin bed.	Air in sample and/or buffer.	Ensure that the volume in the sample vial is sufficient and that no air is injected.
		Degas the buffer and allow it to reach a temperature of +15 to +25°C prior to equilibration of the column.
The buffer does not flow easily through the columns.	High back pressure.	Reduce the flow rate.
	Impurities from the samples may have clogged the column.	Use only particle-free filtered IgG or albumin solutions as samples and controls. Plugging clots on the filter element in the beige column guard connector can be removed when the column is connected in the opposite direction, flushed with 5 volumes Buffer B (flow rate 0.5 mL/min), and then connected in the correct direction again.
Inefficient binding of the target protein to the resin within the columns.	Suboptimal buffer conditions during the binding step.	Use the buffers specified in these Instructions for Use and check the pH.
	FcRn functionality may decrease after >100 injections or after inappropriate treatment, such as harsh buffer conditions with wrong pH, detergents, or too strong or too low salts.	Check the function of the column using the FcRn IgG Control following the protocol in the Instructions for Use.
		Replace the column after 100 injections or if the functionality was damaged by inappropriate treatment.
Inefficient or no elution of the target protein.	The target protein multimerizes and binds more avidly to the resin.	Wash the column with multiple column volumes of Buffer B and repeat negative control.
	Target protein has a biotin label or affinity to streptavidin.	Saturate column with biotin before running samples.
	The target protein precipitates during a pH shift elution.	Wash the column with multiple column volumes of Buffer B and repeat negative control.
Separation of target proteins suboptimal or not as expected.	Suboptimal buffer conditions during the elution step.	Check NaCl/osmolality and pH of buffers.
		Check flow rate and temperature.
		Check that pH gradient is applied correctly and gradient mixer in the Liquid Chromatography System works properly.
	Protein interactions in sample composition may influence the elution profile.	Analyze target proteins individually with the FcRn Affinity Column and overlay the different elution profiles to decouple interaction effects in complex mixtures.
	The target protein may be degraded.	Analyze integrity of target protein with additional methods (SDS-PAGE, mass spectrometry, etc.).
	The resin binding capacity is limiting.	Do not inject more than 100 µg IgG or 500 µg BSA.
		Injection volume should not exceed 80 µL.
	When analyzing BSA on a new unconditioned column, in some cases during the first few runs, the albumin may be bound also nonspecifically to the matrix, resulting in a non-reproducible elution profile.	Before analysis of BSA on a new column, saturate the column once using 30 mL of a solution of 5 mg/mL BSA in binding Buffer A (flow rate 0.5 mL/min), and then wash the column with two buffer gradient runs, see section **Protocol, Step 6**.

5. Additional Information on this Product

5.1. Test Principle

Functional characterization of monoclonal antibodies and serum albumin

The neonatal Fc receptor (FcRn) is important for the metabolic processing of IgG antibodies *in vivo*. Analysis of the interaction between FcRn and IgG *in vitro* allows for assessment of the structural and functional integrity of IgG with regard to impact on the pharmacokinetics *in vivo*. Monoclonal IgG antibodies are used for a rapidly growing range of therapeutic applications for various diseases.

Analytical FcRn column Gen2 chromatography can be used for:

- Preclinical research and pharmaceutical development of drug candidates.
- Control of consistent product quality in the manufacture of therapeutic antibodies.
- Characterization of the pharmacokinetic and pharmacodynamic properties of antibodies.
- Analytics of antibody aggregation, deamination, oxidation, or other posttranslational modifications affecting the FcRn binding.

Chromatography on immobilized FcRn allows for differentiation of IgG variants by peak pattern and retention time profile. Differences in the FcRn chromatographic properties of IgG variants have been shown to correlate to different pharmacokinetic properties in transgenic mice (Schlothauer T, et al, 2013). FcRn chromatography enables a powerful and efficient method for characterization of monoclonal IgG preparations which cannot be done by only one simple method, such as surface plasmon resonance analysis.
Furthermore, FcRn also has an affinity to serum albumin (Sand KMK, et al, 2015), and the FcRn chromatography can be used to characterize serum albumin preparations, for example, for detection of oxidized or aggregated variants.

Test principle

The method for IgG antibody characterization is based on the affinity of immobilized FcRn to the Fc part of the IgG. The affinity is modulated by pH; IgG is bound to the receptor at pH 6 and released by increasing the pH up to 7.4 or higher, mimicking physiological conditions (binding in the endosome, release in the blood). Different IgG Fc subtypes, such as oxidized methionine, oligomerization, glycosylation, or charge variants can be differentiated by their distinct elution patterns.

5.2. References

- Schlothauer T, et al. Analytical FcRn affinity chromatography for functional characterization of monoclonal antibodies. Mabs. 2013;5(4):576-586.
- Sand KMK, et al. Unraveling the Interaction between FcRn and Albumin: Opportunities for Design of Albumin-Based Therapeutics. Front Immunol. 2014;5:682.
- Stracke J, et al. A novel approach to investigate the effect of methionine oxidation on pharmacokinetic properties of therapeutic antibodies. Mabs. 2014;6(5):1229-1242.

5.3. Quality Control

FcRn Affinity Columns Gen2 are checked for correct column packing, and the resin is functionally tested for separation of IgG variants.

6. Supplementary Information

6.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

6.2. Changes to previous version

First version.

6.3. Ordering Information

Product	Pack Size	Cat. No.
Reagents, kits		
FcRn IgG Control	1 mL	09 494 804 001

6.4. Trademarks

All product names and trademarks are the property of their respective owners.

6.4. License Disclaimer

Consult product detail pages at **custombiotech.roche.com** for patent license limitations, if available.

6.5. Regulatory Disclaimer

For use in quality control / manufacturing process only.

6.6. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

6.7. Contact and Support

For additional documentation such as certificates and safety data sheets, please visit **documentation.roche.com**.

Your Roche CustomBiotech Customer Service:

Europe, Middle East, Africa and Latin America
Roche Diagnostics Deutschland GmbH
Phone +49 621 759 8580
Fax +49 621 759 6385
mannheim.custombiotech@roche.com

United States
Roche Diagnostics Corporation
Phone +1 800 428 5433 (toll free)
Fax +1 317 521 4065
custombiotech.ussales@roche.com

Canada
Roche Diagnostics
Phone +1 450 686 7050
Fax +1 450 686 7012
custombiotech.can@roche.co

Japan
Roche Diagnostics K.K.
Phone +81 3 6634 1046
Fax +81 3 5479 0585
japan.custombiotech@roche.com

Asia Pacific
Roche Diagnostics Asia Pacific Pte. Ltd.
Phone +65 6371 6638
Fax +65 6371 6601
apac.custombiotech@roche.com

0622.09652604001 ⓒ

Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany