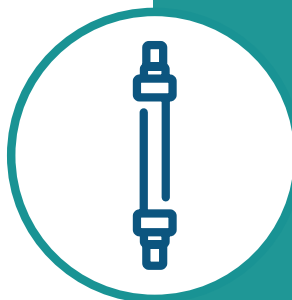




TOSOH



# FcR affinity chromatography of defined Fc N-glycoforms



**Analysis of antibodies**

TOSOH BIOSCIENCE

**SEPARATION  
& PURIFICATION**

CONNECTING MINDS.  
TOUCHING LIVES.

## Your Challenge

- ▶ You need a quick method to estimate mAb glycoforms and ADCC activity.
- ▶ You already use FcR affinity chromatography and need details on what you separated.

## Our Solution

TSKgel FcR-IIIA-NPR affinity chromatography column

- ▶ separate mAbs according to glycan pattern

What was done?

- ▶ Native mAbs and their defined Fc N-glycoforms were separated by affinity chromatography.

What was the result?

- ▶ Retention times of glycoforms (e.g. G0, G1) were related to peaks in native mAb separation.

**Analysis of defined mAb Fc N-glycoforms by FcR affinity HPLC correlated with reported Fc-receptor affinity-glycan relation and allowed for correlating elution times to specific glycoforms.**

## Your Benefit

**Use FcR affinity HPLC as a fast orthogonal method for ADCC or glycan assays**



# Use of defined Fc N-glycoforms to develop a rapid HPLC method for ADCC and mAb glycoform analysis

## Introduction

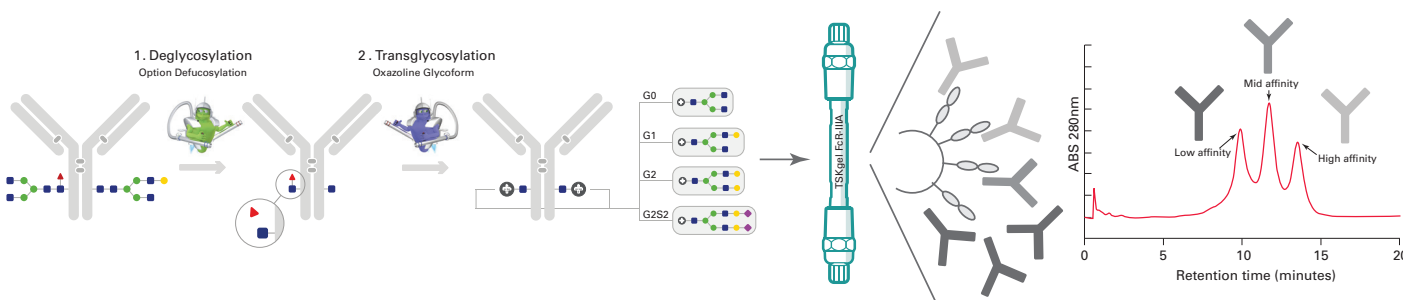
Antibodies contain two regions, the fragment antigen binding (Fab), which specifically binds a target molecule, and the fragment crystallizable (Fc), which confers immunologic functions and impacts serum half-life. These Fc-related activities are regulated by the interaction of the Fc part with Fc receptors on cells of the immune system. One such receptor is FcγRIIIa, expressed by natural killer cells and responsible for cytokine production and lysis of antibody-coated cells. The affinity to the receptor and, thus, cellular activities are impacted by the N-glycosylation of the antibody Fc part, where the core fucosylation has been reported to decrease the affinity to the FcγRIIIa receptor. In contrast, an increased level of galactosylation has been shown to increase the affinity.

In this application note, we investigated the separation of antibodies with defined Fc N-glycans on the TSKgel® FcR-IIIa-NPR HPLC column to evaluate changes in FcγRIIIa affinity related to Fc N-glycan structure. To generate antibodies with defined Fc N-glycans, a transglycosylation technology for Fc-specific N-glycan remodeling of human IgG was used (TransGLYCIT™, Genovis). The TSKgel FcR-IIIa-NPR HPLC column employs a recombinant version of the FcγRIIIa receptor and separates antibodies based on the affinity to the receptor in one single HPLC run without the need for labor- and cost-intensive technologies (Figure 1).

## Experimental Conditions

Column:	TSKgel FcR-IIIa-NPR
Mobile phase:	A: 50 mmol/L Na citrate, 150 mmol/L NaCl, pH 6.5 B: 50 mmol/L Na citrate, 150 mmol/L NaCl, pH 4.5
Gradient:	0–10 min: 0% B; 10–50 min: 0–100% B, 50–60 min: 100–0% B
Flow rate:	0.375 mL/min
Temperature:	20 °C
Injection vol.:	10 µL
Detection:	UV @ 280 nm
Sample conc.:	1 mg/mL, except native antibodies 2 mg/mL
Samples:	Trastuzumab, Cetuximab Antibody sample preparation using the following products according to the manufacturer's instructions:
	<ul style="list-style-type: none"> <li>• TransGLYCIT™ Remodeling with the G0, G1, G2, and G2S2 glycoforms (Genovis)</li> <li>• TransGLYCIT™ Remodeling Afucosylated with the G0, G1, G2, and G2S2 glycoforms (Genovis)</li> <li>• GlycINATOR® deglycosylation (Genovis)</li> <li>• GlycINATOR® + FucosEXO™ 16 deglycosylation and afucosylation (Genovis)</li> </ul>

**Figure 1.** Experimental overview. Monoclonal antibodies (mAbs) were remodeled to carry defined Fc N-glycans and subsequently analyzed on the FcR-IIIa-NPR affinity column to determine FcγRIIIa receptor affinity.



For Fc-specific N-glycan remodeling using TransGLYCIT, the Fc N-glycans were trimmed to the core GlcNAc using GlycINATOR, an IgG-specific immobilized EndoS2 hydrolase active towards all Fc N-glycan structures, including high-mannose, hybrid, complex and bisecting N-glycans. To obtain core afucosylated glycoforms, an immobilized exo- $\alpha$ -1-6 fucosidase, FucosEXO 16, was included to hydrolyze the  $\alpha$ -1-6-linked core fucose from the GlcNAc residue. Subsequently, an engineered EndoS2 glycosynthase was used to catalyze the transglycosylation reaction between one of the oxazoline-reactive G0, G1, G2, or G2S2 glycoforms and the core GlcNAc resulting in antibodies with defined and homogeneous Fc N-glycan structures. As a final step, affinity purification of the antibodies was performed. For LC-MS verification, the antibodies were digested with FabRICATOR (IdeS). The subunits were analyzed by reversed-phase LC-MS on a Bruker Impact II ESI-QTOF MS. The analysis of the native and remodeled mAbs on the FcR-IIIa-NPR HPLC column was performed as outlined above.

## Results and Discussion

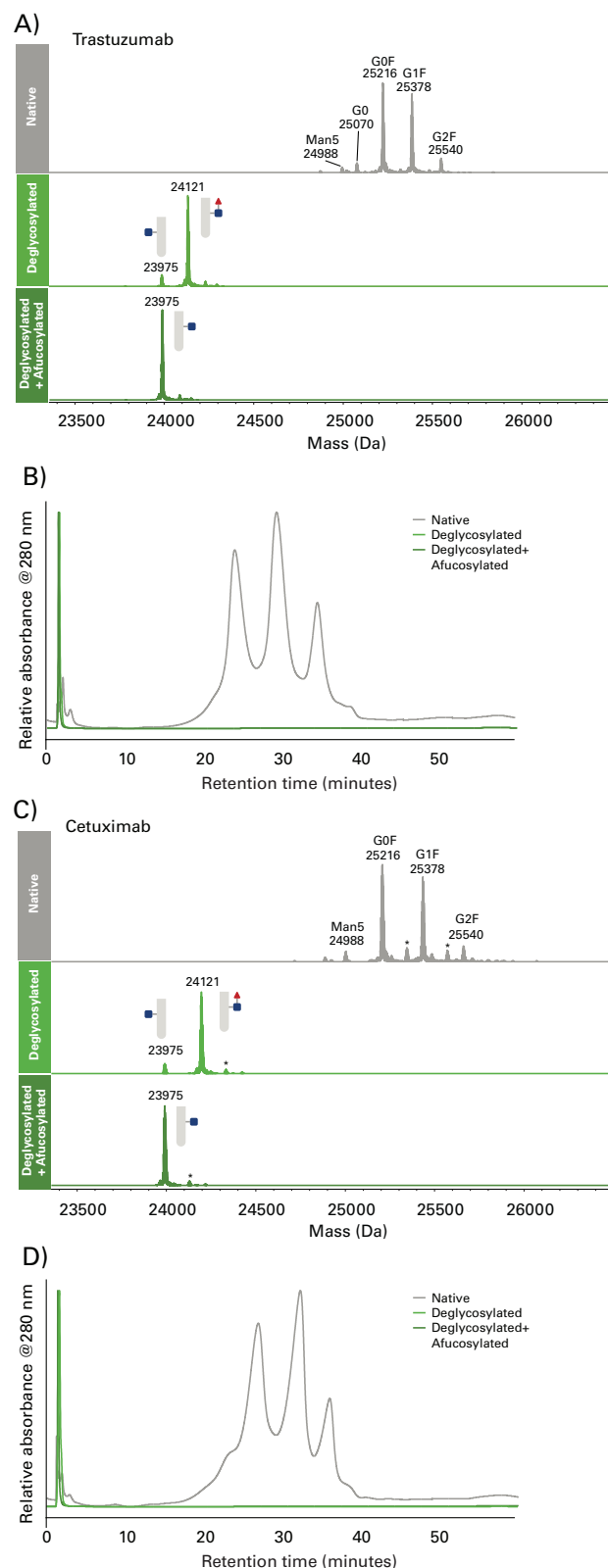
### Impact of glycosylation of native trastuzumab and cetuximab on FcR-IIIa chromatography

The two therapeutic monoclonal antibodies (mAbs), trastuzumab and cetuximab, were analyzed regarding their native Fc N-glycan structures and upon deglycosylation as well as upon deglycosylation and afucosylation. For this purpose, LC-MS of FabRICATOR-digested mAbs (resulting in scFc and F(ab')<sub>2</sub> fragments) was performed to identify glycoforms in the antibody samples: the Fc N-glycans of both trastuzumab (Figure 2a) and cetuximab (Figure 2c) were mainly composed of G0F and G1F, whereas G2F, G0, and Man5 were minor glycoforms. LC-MS analysis of the deglycosylated samples confirmed the complete deglycosylation to the core GlcNAc of the Fc N-glycan. The core GlcNAc was mainly fucosylated in both mAbs, although a smaller portion of afucosylated core GlcNAc was also present. This is explained by the presence of both core fucosylated and afucosylated Fc N-glycans of the originating native mAbs. Upon core afucosylation, the only modification found by LC-MS analysis of the scFc fragments was the GlcNAc residue.

Next, the native mAbs were analyzed regarding their affinity to the Fc $\gamma$ RIIIa receptor by HPLC. Both mAbs eluted from the FcR-IIIa-NPR column as three peaks between 22–38 min corresponding to variants with low, medium, and high affinity to the Fc $\gamma$ RIIIa receptor (Figure 2b and 2d). Deglycosylation resulted in no binding to the FcR-IIIa-NPR column, irrespective of core fucosylation. This indicates that Fc N-glycans are essential for the Fc $\gamma$ RIIIa receptor binding.

**Figure 2.** Structural analysis of Trastuzumab and Cetuximab glycovariants.

Mass spectra of scFc fragments of native, deglycosylated, and deglycosylated + afucosylated trastuzumab (a) and cetuximab (c). The peaks indicated by an asterisk correspond to the main scFc peaks carrying a C-terminal lysine ( $\Delta m = 128$  Da). The corresponding trastuzumab (b) and cetuximab (d) variants were separated on the FcR-IIIa-NPR column to determine the Fc $\gamma$ RIIIa receptor affinity.

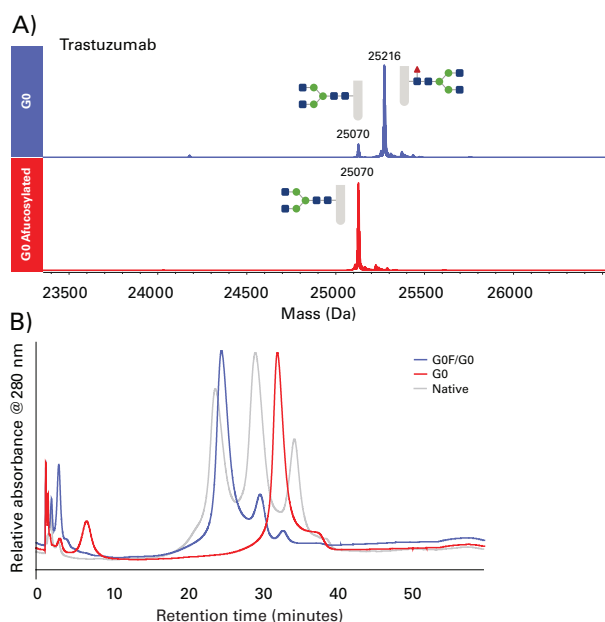


## Impact of core fucosylation on FcR-IIIa affinity chromatography

Since the initial data did not allow correlations between glycoforms and the Fc $\gamma$ RIIIa affinity separation, the homogeneous glycoforms with and without core fucosylation were analyzed as the next step. The G0 glycoform of trastuzumab was selected as an example. Structural analysis of the scFc of the core fucosylated G0 sample showed that in addition to G0F, a small amount of G0 was present, whereas the core afucosylated G0 sample contained only G0 (**Figure 3a**).

These Fc N-glycan-remodeled mAbs were separated on the FcR-IIIa-NPR column. The fucosylated G0 sample (G0F/G0) eluted as three peaks: the first peak was the largest one and overlapped with the low-affinity peak of the native sample (**Figure 3b**), whereas the two minor peaks eluted with the medium affinity peak and between the medium and high-affinity peaks, respectively. In contrast, the core afucosylated G0 sample (G0) resulted in just one major peak with an elution time between the medium and high-affinity peaks of the native mAbs and overlapped with the last eluting peak in the fucosylated G0 sample that contained a small amount of G0.

**Figure 3.** Structural analysis of G0-remodeled trastuzumab. Mass spectra of scFc fragments with and without core fucosylation (a) and Fc $\gamma$ RIIIa affinity separation of remodeled trastuzumab variants (b).



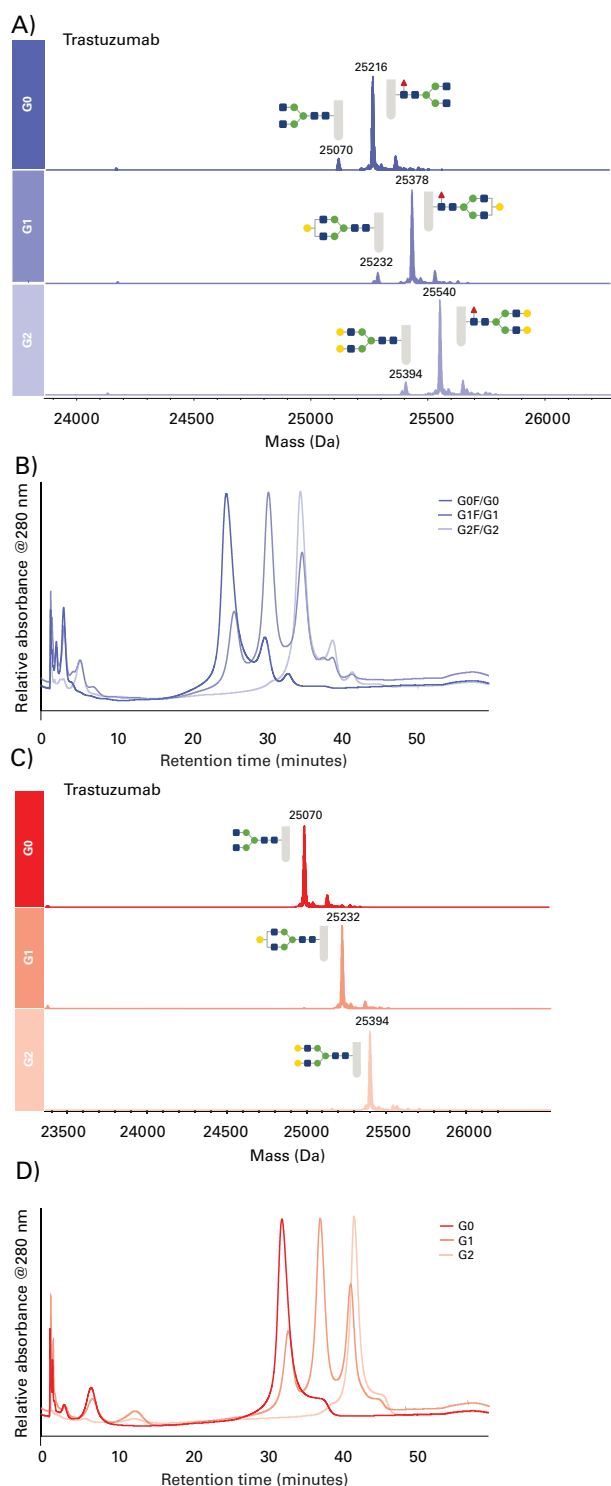
The structural information helped to understand the presence of the three peaks in the fucosylated G0 sample: the antibodies carrying G0F on both heavy chains have the lowest Fc-receptor affinity and elute in the first peak, the antibodies carrying one G0 and one G0F elute in the second peak, and the antibodies carrying two G0 in the third peak. For both antibodies and all glycoforms tested (G0, **Figure 3**; G1, G2 in **Figure 4**; cetuximab not shown), we observed shifts to later elution times upon core afucosylation corresponding to higher affinities for the Fc $\gamma$ RIIIa receptor.

## Impact of galactosylation on FcR-IIIa affinity chromatography

Next, the impact of galactosylation on the separation by FcR-IIIa affinity chromatography was investigated for the remodeled trastuzumab. First, the structures of the remodeled samples were verified using LC-MS. In accordance with the previous experiments, the core fucosylated G0, G1, and G2 samples contained mainly G0F, G1F and G2F, respectively, and a minor afucosylated amount (G0F/G0, G1F/G1, G2F/G2; **Figure 4a**), whereas the corresponding core afucosylated G0, G1, and G2 samples contained only G0, G1, and G2, respectively.

The addition of terminal galactoses resulted in different elution profiles on the FcR-IIIa column (**Figure 4b**): the higher the galactose levels, the later eluting peaks. Interestingly, the G1 glycoform (G1F/G1 and G1) resulted in three separate peaks despite just one mass in the LC-MS analysis. A possible explanation may be that G1 glycan is present as two isoforms. The terminal galactose may either be linked to the  $\alpha$ 1-3 mannose arm or the  $\alpha$ 1-6mannose arm. It has previously been shown that the Fc $\gamma$ RIIIa receptor has a higher affinity towards the G1 glycoform galactosylated on the  $\alpha$ 1-6 mannose arm than the one on the  $\alpha$ 1-3 mannose arm [1]. This assumption will be verified in subsequent studies.

**Figure 4.** Structural analysis of G0-, G1- and G2-remodeled trastuzumab. Mass spectra of the mAb-derived scFc fragments with (a) and without (c) core fucosylation and FcγRIIIa affinity separation of the remodeled trastuzumab variants with (b) and without (d) core fucosylation.



## Conclusion

Using antibodies with defined and homogeneous Fc N-glycoforms, we demonstrated the impact of the Fc N-glycan structure on FcγRIIIa binding (Table 1). The data obtained with the FcR-IIIa-NPR column were in accordance with previously published data on how FcγRIIIa affinity is altered with the Fc N-glycosylation of mAbs [2]. This application demonstrates the efficacy and proof of concept of FcγRIIIa chromatography for a fast orthogonal analysis to evaluate mAb ADCC activity, potentially for early cell line development, bioreactor modeling, and lot-to-lot comparability of therapeutic antibodies.

We kindly thank Genovis, particularly Andrea Persson, for generating the data and working on the application note draft.

**Table 1.** Summary of FcγRIIIa affinity of Fc N-glycan remodeled antibodies. Peaks 1-3 refer to the peaks observed for the native trastuzumab and cetuximab.

Peak in native mAb	Glycoforms Included
Peak 1 (low affinity)	G0F, G1F
Peak 2 (medium affinity)	G1F
In-between peak 2 and 3 (medium-high affinity)	G0, G2F
Peak 3 (high affinity)	G1F, G2F
Later elution than peak 3 (Highest affinity)	G1, G2

## References

- Aoyama M et al. (2019) Effects of terminal galactose residues in mannose  $\alpha$ 1-6 arm of Fc-glycan on the effector functions of therapeutic monoclonal antibodies, mAbs, 11:5, 826-836, DOI: [10.1080/19420862.2019.1608143](https://doi.org/10.1080/19420862.2019.1608143)
- Cambay F et al. (2020) Impact of IgG1 N-glycosylation on their interaction with Fc gamma receptors. Current Research in Immunology: 1, 23-37, 2020. DOI: <https://doi.org/10.1016/j.crimmu.2020.06.001>

## Featured Product

Part #	Description	Column dimensions
0023513	TSKgel FcR-IIIa-NPR	4.6 mm ID x 7.5 cm L

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