# N-glycosylation Customization of Rituximab using *FastPharming*™ system

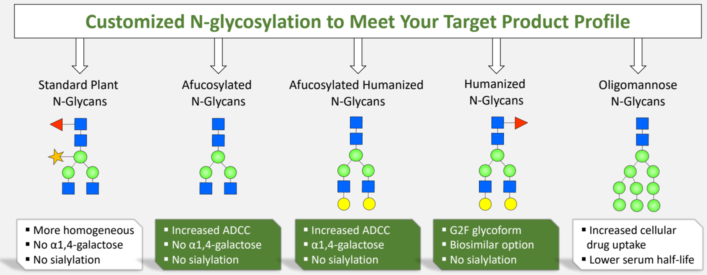
**Roman Subbotin, Sreenath Palle, Jasmine Deng, Zhongjie Ren, Yo Ng, Phyllis Loupot, Vally Kommineni, Brian Berquist and Sylvain Marcel***

iBio CDMO, LLC, 8800 Health Science Center Parkway, Bryan, TX 77807, USA
*Correspondence: smarcel@ibiocmo.com

## ABSTRACT

N-glycosylation is known to affect the biological activity of human therapeutics such as anti-cancer monoclonal antibodies, blood factors, or lysosomal enzymes. Over the last decade, several methods have been developed to enable the engineering of N-glycosylation profiles present on certain drugs. For instance, Chinese Hamster Ovary (CHO) cells or the yeast *Pichia pastoris* have been modified to produce afucosylated antibodies to enhance the Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) of the drug candidates. However, the availability of such cell lines for development or commercial purposes remains limited or expensive. iBio's *FastPharming*$^{TM}$ technology uses a protein expression platform that combines the transfection of *Nicotiana benthamiana* plants at pilot and commercial scales and a unique set of glycan engineering techniques. Using rituximab as a model protein, we demonstrated the power of glycosylation engineering by design that *FastPharming* offers.

We first generated G0 rituximab using a transgenic plant engineered to remove core fucose. With a second transgenic plant expressing the human α1,4-galactosyltrasferase, G1 and G2 rituximab glycoforms were produced. Finally, we modified our plant transfection method to introduce the mannosidase I inhibitor kifunensine to generate rituximab decorated with oligomannose residues. All glyco-engineered rituximab proteins show enhanced ADCC activity in vitro without affecting the protein ability to bind CD-20 receptor.

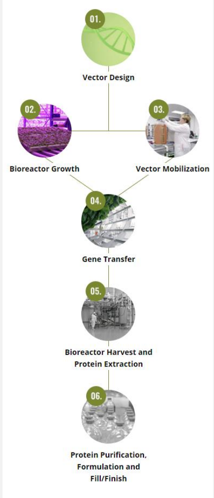


Customized N-glycosylation to Meet Your Target Product Profile

| Standard Plant N-Glycans | Afucosylated N-Glycans | Afucosylated Humanized N-Glycans | Humanized N-Glycans | Oligomannose N-Glycans |
|---|---|---|---|---|
| ▪ More homogeneous<br>▪ No α1,4-galactose<br>▪ No sialylation | ▪ Increased ADCC<br>▪ No α1,4-galactose<br>▪ No sialylation | ▪ Increased ADCC<br>▪ α1,4-galactose<br>▪ No sialylation | ▪ G2F glycoform<br>▪ Biosimilar option<br>▪ No sialylation | ▪ Increased cellular drug uptake<br>▪ Lower serum half-life |

**Figure 1**. The *Nicotiana benthamiana* plant expression platform provides a range of custom N-glycosylation adapted to enhance utility in biotherapeutic applications.

## Rituximab Expression using *FastPharming* Technology



### Rituximab expression process in *Nicotiana benthamiana*

**01.** The Gene of Interest (GOI) is inserted into iBio's proprietary plant expression vectors

**02.** Plants are grown hydroponically using chemically defined nutrients in vertical farming rooms

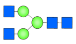
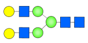
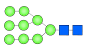
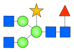
**03.** Plant expression vectors are mobilized into *Agrobacterium tumefaciens* and amplified in bioreactors

**04.** GOI is transferred to plant cells during a transfection process designed for manufacturing scale

**05.** Protein of interest is expressed transiently in plants for 6-8 days before being harvested for purification

**06**. Clarified plant protein extract enters the downstream process train in classified clean rooms for product purification and vialing

**Table 1**. Rituximab expression in different plants or plant treatments.

| Plant line | ΔXT/FT | ΔXT/FT + GalT | Treated with kifunensine | Wild type |
|---|---|---|---|---|
| Expected Major Protein Glycoform |  |  |  |  |
| Expression mg/kg biomass | 351 ±25 | 375 ±32 | 285 ±51 | 287 ±64 |

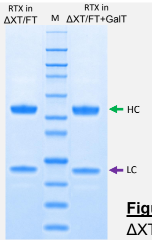


**Figure 2**. SDS-PAGE (reduced) of purified rituximab (RTX) produced in ΔXT/FT and ΔXT/FT+GalT lines.

## N-glycan profiling of customized rituximab

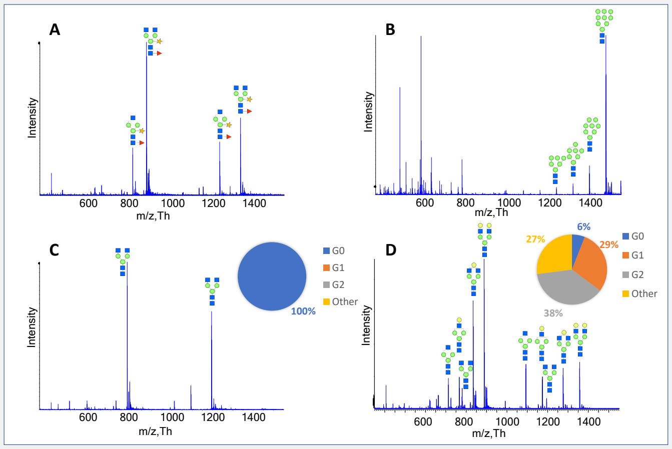


**Figure 3**. Glycopeptide MS spectra from four rituximab samples: **A**. Rituximab sample produced in wild type plants decorated with plant complex N-glycans; **B**. Rituximab sample from kifunensine-treated plants decorated with high-mannose glycans; **C**. Rituximab sample from ΔXT/FT plants decorated with G0 glycan; **D**. Rituximab sample from ΔXT/FT + GalT plants decorated with G1 and G2 glycans.

- Rituximab derived from ΔXT/FT plants show homogeneous G0 glycoform
- ΔXT/FT + GalT plants generated G1/G2 glycoforms as in FUT8$^{-/-}$ CHO knockout cell lines

## Material and Methods

***Material***

Seeds of *Nicotiana benthamiana* wild type, C105 ΔXT/FT and C105-GalT transgenic lines were grown hydroponically under LED lights at the iBio manufacturing facility, Bryan, Texas. The rituximab sequence was obtained from DrugBank (Accession DB00073).

***Methods***

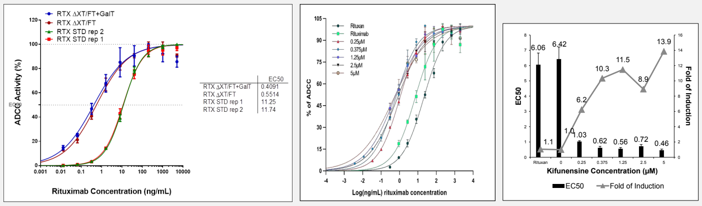
Cloning and Protein Expression: Rituximab heavy and light chain genes were cloned into iBio proprietary vectors and mobilized into *Agrobacterium tumefaciens GV3101:pMP90* and transferred to 4-week-old *N. benthamiana* plants by Agroinfiltration as described in Kommineni et al., 2019. For Kifunensine treatments, concentration of 5μM, 2.5μM, 1.25μM, 0.75μM, 0.375μM and 0.25μM were used in the infiltration solution.

Protein Purification: Infiltrated leaves were harvested at 6 or 7dpi and total soluble proteins were extracted in 50mM sodium phosphate, 150mM NaCl, 5mM EDTA, 60mM ascorbic acid). Rituximab was capture by protein A chromatography as described in Bennett et al., 2018. Elution fractions were analyzed on a 4-12% Bis-Tris gradient NuPAGE$^{TM}$ gel under reducing and non-reducing conditions and stained using the GenScript eStain$^{TM}$ L1 Protein Staining System.

Glycopetide Analysis by LC-MS: Sample preparation was performed following standard procedure for proteomic sample preparation. All samples were reduced with DTT, alkylated with iodoacetamide and were subjected to tryptic proteolysis. Resulting peptide mixtures were desalted using in-house prepared C18 SPE and were subjected to LC-MS/MS analysis. Peptides were separated using 15min 5 to 95% acetonitrile gradient on C18 column at 40°C. Eluted peptides were ionized at the Turbo V source (SCIEX) at 5kV and were analyzed using SCIEX 5600 TripleTOF instrument. Standard top 20 IDA experiment was performed for protein identification. Glycopeptide ions were identified using 204 Th diagnostic MS2 fragment peak and by matching MS1 ion masses to theoretical rituximab glycopeptide masses.

Binding and Cell-Based Assay: Rituximab binding to target cells Wil2-S was determined by flow cytometry as described in Kommineni et al., 2019. Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) assay was performed using the Promega ADCC Reporter Bioassay with Wil2-S cells and FcγRIIIa receptor, V158 variant effector cells. GraphPad prism software was used to plot normalized Relative Luminescence Units (RLU) against rituximab concentration in Log10 form. The half maximal Effective Concentrations (EC50) were calculated from non-linear regression curves.

## ADCC activity and CD-20 binding of customized rituximab



| | EC50 |
|---|---|
| RTX ΔXT/FT+GalT | 0.4091 |
| RTX ΔXT/FT | 0.5514 |
| RTX STD rep 1 | 11.25 |
| RTX STD rep 2 | 11.74 |

**Figure 4**. In vitro ADCC activity of different rituximab glycoforms compared to rituximab standard.

- Superior ADCC was confirmed with all afucosylated rituximab proteins
- Rituximab glycosylation profiles did not affect CD-20 binding efficiency

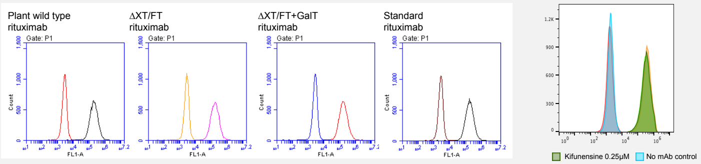


**Figure 5**. Rituximab binding affinities to CD-20 receptor on Wil2-S target cells analyzed by flow cytometry. Peaks on the left represent mIgG2a isotype control while peaks on the right represent binding of rituximab samples.

1- Strasser et al. (2008) Generation of glycol-engineered *Nicotiana benthamiana* for the production of monoclonal antibodies with homogeneous human-like N-glycan structures. ***Plant Biotech. J.*** **6:**392-402
2- Schneider et al. (2015) Characterization of plants expressing the human β1,4-galactosyltransferase gene. ***Plant Physiol. Biochem.*** **95:**39-47
3- Kommineni et al. (2019) In vivo glycan engineering via the mannosidase I inhibitor (kifunensine) improves efficacy of rituximab manufactured in *Nicotiana benthamiana* plants. ***Int. J. Mol. Sci.*** **20(1)**:194
4- Bennett et al. (2018) Implementation of glycan remodeling to plant-made therapeutic antibodies. ***Int. J. Mol. Sci.*** **19(2)**:421