Fc-fusion Cell Line Development: Expression and Analytical Strategies

By Leon Pybus[1], Fay Saunders[1], Devika Kalsi[1], Jeffrey Keen[1], Jessica Kane-Fidgeon[1], Daniel Sharkey[1], Yuta Murakami[2], Tatsuya Matsuura[2], Kenta Seko[2]

[1]FUJIFILM Diosynth Biotechnologies, Teesside, UK
[2]FUJIFILM Corporation, Ashigarakami-gun, Kanagawa, Japan

Following the clinical and commercial success of monoclonal antibodies, Fc-based therapies have captured substantial attention in the biopharmaceutical industry; however, their inherent complexity continues to present manufacturability challenges in both upstream and downstream processes. We describe high titer expression of Fc-fusion proteins using FUJIFILM Diosynth Biotechnologies' Apollo™ X platform coupled with integrated quality assessments, enhancing our support for clients through the predicted growth in Fc-fusion clinical applications.

Fc-fusion Proteins: Enhanced Pharmacokinetics and Therapeutic Activity

Fc-fusion proteins are engineered to contain an immunoglobin Fc domain that is directly linked to the protein or peptide of interest (Fig. 1),[1] endowing these hybrid candidates with beneficial pharmacokinetic properties such as increased plasma half-life, stability, avidity, and potency,[2] and enhancing their therapeutic activity against a wide range of pathologies.[3,4]

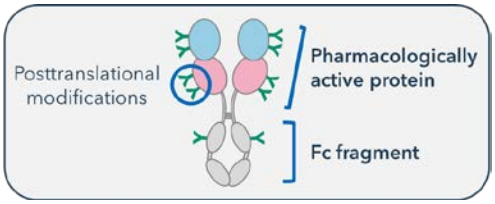


Figure 1. Basic structure of Fc-fusion proteins including the Fc-domain. Adapted from *Duivelshof et al 2021 (J Sep Sci).*

Although monoclonal antibodies (mAbs) remain the top selling biologics globally,[5] clinical applications of commercial Fc-based therapies continue to grow (Table 1). In Europe and the US, manufacturing demand for clinical and commercial antibody fusion products is expected to grow at an annual rate of ≥ 3% (Table 2), with 30-40% of this demand expected to be outsourced to CDMOs.[6]

Table 1. Fourteen Fc-fusion proteins have been approved in the European Union and United States, and numerous Fc-fusion products are currently in pre-clinical and clinical development.[1,7]

Product	Company	Fc-fused Protein	2021 sales ($M)
Eylea	Regeneron Pharmaceuticals Inc.	VEGF Receptors 1 and 2	$ 9,241.40
Trulicity	Eli Lilly and Co.	Glucagon-like peptide 1 (GLP-1)	$ 6,174.90
Enbrel	Amgen Inc.	Tumor necrosis factor receptor (TNFR p75)	$ 5,650.00
Orencia	Bristol Myers Squibb Co.	Cytotoxic T lymphocyte antigen-4 (CTLA-4)	$ 3,306.00
Eloctate | Elocta (EU)	Sanofi SA (Bioverativ Inc.)	Factor VIII	$ 1,126.80
AlprolIX	Sanofi SA (Bioverativ Inc.)	Factor IX	$ 694.80
Strensiq	AstraZeneca plc (Alexion)	Catalytic domain of human TNAP	$ 575.50
Eticovo | Benepali (EU)	Samsung Bioepis	Tumor necrosis factor receptor (TNFR p75)	$ 561.30
Reblozyl	Merck & Co. Inc.	Activin receptor type 2B (ACVR2B)	$ 551.00
Erelzi	Novartis AG (Sandoz)	Tumor necrosis factor receptor (TNFR p75)	$ 170.00
Zaltrap	Regeneron Pharmaceuticals Inc.	VEGF Receptors 1 and 2	$ 91.70
Nulojix	Bristol Myers Squibb Co.	Cytotoxic T lymphocyte antigen-4 (CTLA-4)	$ 90.00
Arcalyst	Kiniksa Pharmaceuticals Ltd.	IL-1 receptor component, accessory protein	$ 40.70
Nepexto (EU)	Viatris Inc.	Tumor necrosis factor receptor (TNFR p75)	$ 50.00

Table 2. Estimated production growth of Fc-based biologics.

Fc-Fusion Product (1,000 L/Year)	2022	2023	2024	2025	2026
Commercial	261	267	277	290	295
BLA/MAA	0.03	0.1	0.1	0.2	0.3
Phase 3	-	-	-	-	0.02
Phase 2	-	-	-	-	1
Phase 1	-	-	-	-	-
Clinical	13	15	16	17	19
Total	274	282	293	307	315

However, despite significant advances in fusion protein technology, progress in bench-to-bedside development of Fc-based therapies remains hindered. Poor physicochemistry can lead to failure of candidates in clinical trials,[8,9] and optimization of therapeutic outcomes via Fc fragment modification is still an area of intense investigation.[8] Glycosylation plays a particularly important role in the stability, therapeutic potential, and immunogenicity of mAbs and Fc-fusion products.[10] Assessing glycosylation of Fc-fusion candidates throughout the production process is vital to ensure product stability and proper safety and efficacy profiles.

Fc-fusion manufacturability and quality challenges

The unique structure of Fc-fusion proteins can lead to expression, purity, stability, and quality assessment challenges that must be addressed to meet increasing demand.

Production yields for fusion proteins can be lower than those of mAbs due to less efficient translation or increased susceptibility to proteolytic degradation during production.[11] Incomplete or heterogeneous glycosylation can also affect functionality, conformation and/or stability of the fusion product, further complicating manufacturability.[8] While most therapeutic mAbs contain glycosylation sites in the Fc region and (in some cases) the Fab region, glycosylation of Fc-fusion proteins can also occur in the fusion partners, resulting in diverse and differential glycosylation patterns that have the potential to more significantly impact Fc-fusion product pharmacokinetics, efficacy, and safety profiles.[11]

The structural diversity and complex glycosylation of Fc-fusion proteins can also present analytical characterization challenges, often requiring product-specific methods in place of available generic or platform technologies that are typically used to assess the quality of biologics.[4]

Apollo™ X: Bridging the Productivity Gap

Expression in mammalian systems under optimal bioreactor[12,13] and feed supplementation conditions can directly impact Fc-fusion protein conformation, folding, and post-translational modifications, allowing for increased specific productivity, decreased aggregation, and optimal glycosylation.[14]

Successful implementation of these strategies requires extensive technical knowledge and practical know-how. FUJIFILM Diosynth Biotechnologies' Apollo™ X mammalian expression system combines streamlined process development and manufacturability assessment with biomanufacturing-ready cell lines. This allows FUJIFILM Diosynth Biotechnologies to take biopharmaceuticals from pre-clinical to commercial production efficiently and rapidly.

FEATURES

Host cell line

- DG44-derived CHO host cell line
- Adapted to tailor-made, chemically-defined basal medium and suspension culture
- Superior growth characteristics and expression capability
- Fully characterized cGMP cell bank

Expression Vector

- Double gene vector
- DHFR-based selection system
- Proprietary leader sequence and codon optimization for stable expression and efficient secretion of the target protein

Process

- Robust cloning strategy
- Automated productivity screening and cell isolation remove CLD bottlenecks for high specificity and quality
- Multi-candidate screening and manufacturability assessment package identifies lead candidates and de-risks development process

Apollo™ X was created using an adapted evolution strategy[15] to exploit intrinsic host cell line heterogeneity and select for superior biomanufacturing potential. A novel workflow aids in the generation of high-quality clonal cell lines with >99% probability of monoclonality in a single round of cloning, balancing speed and quality in cell line development.

The stable and clonal cell lines generated using the Apollo™ X system are suitable for use in both fed-batch and continuous culture, supporting an intensified DNA-to-research-cell-bank timeline of approximately 10 weeks, and achieving product concentrations of >5 g/L for mAbs (Fig. 2). The process is simple, robust, and scalable for every bioreactor size, and can be used for mAbs and next generation biotherapeutics such as bispecific antibodies and Fc-fusions.

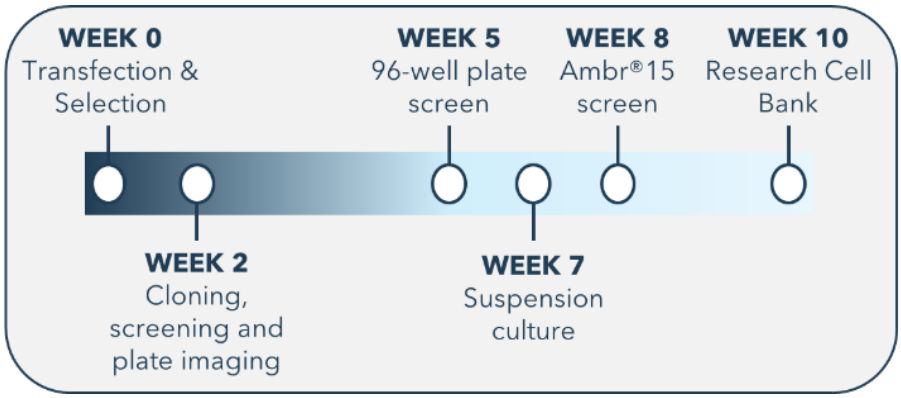


Figure 2. CLD process timeline. Product concentrations of >5 g/L are typically achieved 10 weeks after transfection.

Here, we test the platform's ability to express multiple Fc-fusion products, as well as the quality assessment capabilities of an abbreviated analytical package.

Selecting relevant Fc-fusion proteins for productivity assays

A survey of Fc-fusion manufacturing trends revealed that there are 26 types of Fc-fusion molecules currently approved or in clinical development (Table 3). Analysis of their properties using information contained in the public monoclonal antibodies database IMGT/mAb-DB indicated that 65% of these fusion products contain an IGHG1 Fc fragment. Additionally, these proteins are typically 50-120 kDa in size, are mostly homodimers, and have isoelectric points (pIs) that range between 5-9 (Fig. 3).

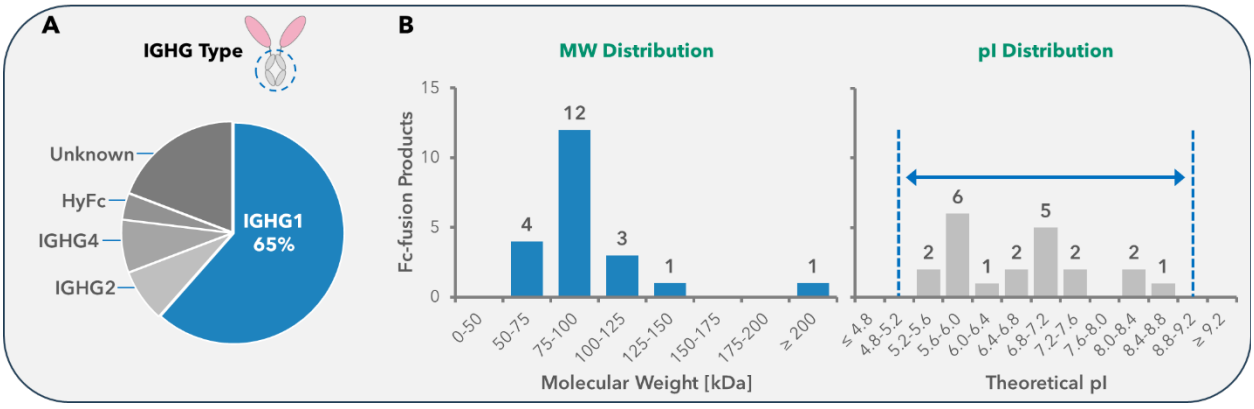


Figure 3. Physicochemical properties of Fc-fusion products currently approved or in clinical development.

Based on these findings, we set out to verify the productivity of Fc-fusion molecules that contain an IGHG1 Fc fragment and form bilaterally symmetrical dimer (homodimer) structures in Apollo™ X.

Table 3. Characteristics of Fc-fusion-type therapeutics currently approved or in clinical development (Source: Evaluate Ltd 2021).

Product	Number of Products	Fc-fused Protein	Fc Fragment Type	Structure
Abatacept	1	CTLA4	IGHG1	Homo dimer
Acazicolcept	2	ICOSLG	IGHG1	Homo dimer
Aflibercept	15	VEGFR-1 – VEGFR-2	IGHG1	Homo dimer
Asunercept	2	TNFRSF6	IGHG1	Homo dimer
Atacicept	2	TNFRSF13B	IGHG1	Homo dimer
Belatacept	1	CTLA4	IGHG1	Homo dimer
Blisibimod	2	peptide 16-mer – peptide 19-mer	IGHG1	Homo dimer
Efanesoctocog alfa	3	F8 – VWF – XTEN	IGHG1	—
Efavaleukin alfa	1	IL2	IGHG1	Homo dimer*
Efbemalenograstim alfa	1	CSF3	IGHG2	Homo dimer
Efepoetin alfa	1	—	HyFc	—
Eflepedocokin alfa	1	IL22	IGHG2	Homo dimer
Efmarodocokin alfa	2	IL22	IGHG4	Homo dimer
Efneptakin alfa	1	—	—	—
Efpegsomatropin	1	GH1 - PEG Linker	IGHG4	Hetero dimer
Eftilagimod alpha	2	LAG3	IGHG1	Homo dimer
Efrenonacog alfa	1	coagulation factor IX	IGHG1	Hetero dimer
ER-004	4	EDA	—	Hexamer
Etanercept	13	TNFRSF1B	IGHG1	Homo dimer
Ontorpacept	1	SIRPA	IGHG1	Homo dimer
Revacept	2	—	—	—
rhTNFR(m)-Fc	1	—	—	—
Rilonacept	1	IL1RAP – IL1R1	IGHG1	Homo dimer
Sotatercept	2	ACVR2A	IGHG1	Homo dimer
Trebananib	2	peptide 14-mer – peptide 14-mer	IGHG1	Homo dimer*
Tulinercept	1	TNFRSF1B	IGHG1	Homo dimer

**N-terminus Fc*

To assess Apollo™ X expression capabilities and evaluate the effects of MW (molecular weight) and pI (isoelectric point) on Fc-fusion protein productivity, we selected seven molecules with varying MW and pI based on amino acid sequence information (Fig. 4, Table 4 below). Productivity results should therefore be representative of a wide range of Fc-fusion proteins that are currently approved for clinical use or still in development.

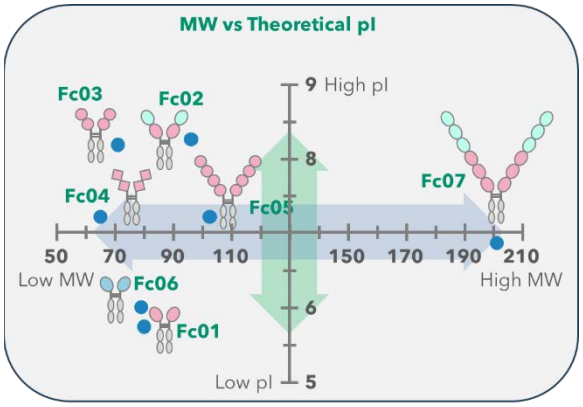


Figure 4. Fc-fusions selected for analysis.

Assessing Fc-fusion productivity

Stable expression cell lines were established using a single gene vector (SGV) for each Fc-fusion product. SGVs were linearized and transfected into Apollo™ X cells via electroporation at uniform amounts to minimize the effect of gene insertion number on productivity. Stable expression pools were generated by selection with MTX and expanded into 125 mL volume non-baffled vented flasks for fed-batch culture. Standard methods were used to assess cell viability, Fc-fusion product concentration, and purification results.

Cell culture was performed using Fc-fusion transfectant pools to assess productivity in the Apollo™ X platform. Six of the seven Fc-fusion products tested reached productivity levels of 1 g/L or more using fed-batch culture of transfectant pools (Table 4); titer is expected to be higher in clonal cell lines. The viable cell density (VCD) peak of each Fc-fusion expressing cell pool was ~40 x 10^6 cells/mL, similar to those attained in mAb production.

Table 4. Fed-batch culture of Fc-fusion proteins selected for productivity assays.

Fc-fusion	Theoretical MW [kDa]	pI	IVCD [x10^6 (day · cells)/mL]	Titer* [g/L]
Fc01	79	5.7	319	1.4
Fc02	97	8.2	312	1.1
Fc03	71	8.1	306	1.2
Fc04	64	7.2	358	2.2
Fc05	102	7.2	274	1.6
Fc06	78	6.0	293	1.6
Fc07	201	6.9	330	0.3

*Titer was measured with CedexBio and corrected by theoretical MW

Examining the effect of Fc-fusion properties on productivity

A preliminary study assessing expression of Fc-fusion proteins of varying size suggested that MW has a significant impact on Fc-fusion productivity. On the other hand, pI was hypothesized to influence degradation and aggregation during cell culture and downstream purification processes. We assessed the effects of MW, pI, and mRNA levels on production of the Fc-fusion proteins selected (Fig. 5).

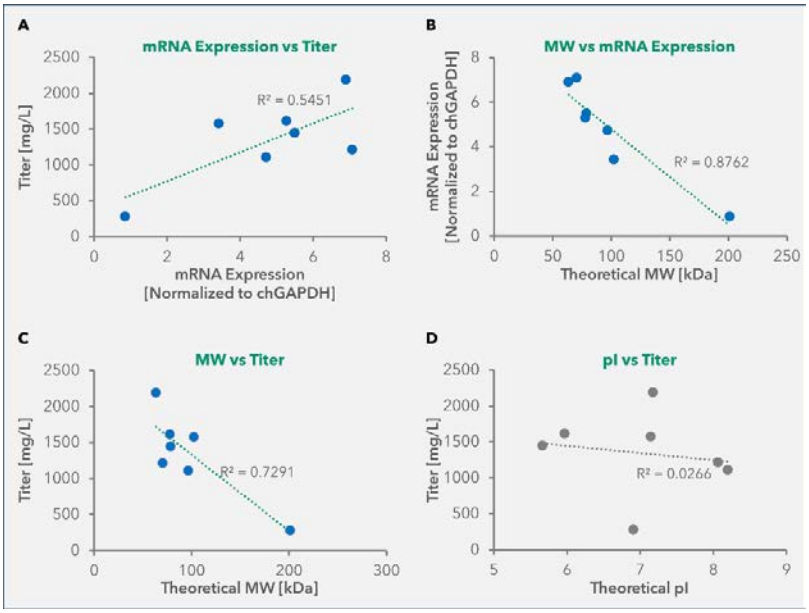


Figure 5. mRNA levels, MW, pI, and Fc-fusion product titer in transfectant pools.

MW and mRNA levels were closely correlated with titer. These results were not unexpected: transcription has long been considered a dominant factor in protein expression control, and correlations between transcript levels and mAb productivity have been observed.[16,17,18,19]

In general, Fc-fusion molecules of ≤ 110 kDa achieved productivities of 1 g/L or more in Apollo™ X transfectant pools. On the other hand, we observed no effect of pI on productivity (Fig. 5D), suggesting that pI-mediated aggregation is not an issue for these Fc-fusions when the Apollo™ X workflow is used, or that it is not at play within the range tested.

Fc02: Clonal cell line development and product analysis

To further explore the Fc-fusion expression capabilities of Apollo™ X and the analytical power of our abbreviated package, we took a closer look at Fc02 productivity and quality assessment. Fc02's inherent structural complexity and posttranslational modifications make it a good candidate to map the possible challenges we may encounter in the production and analysis of Fc-fusion proteins.

Cell line development

48 cell lines expressing Fc02 were generated using the Apollo™ X platform's cell line development (CLD) process and evaluated in the Ambr®15 fed-batch screen over a 14-day period.

Final product concentrations ranged from 3.4 to 4.3 g/L for the 10 highest producers (Fig. 6), suggesting that under clonal conditions, the platform's standard development workflow is sufficient to produce Fc-fusion proteins at titers comparable to those of mAbs.

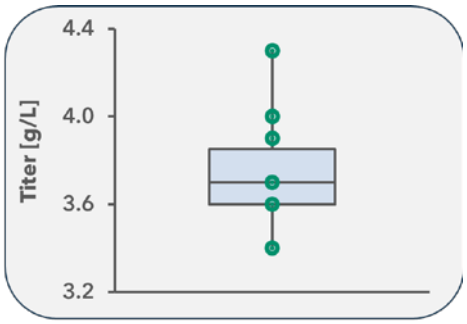
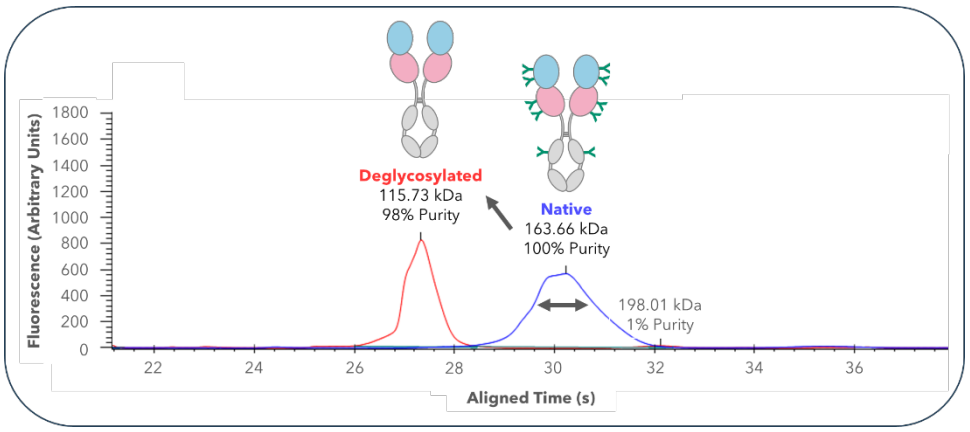


Figure 6. Fc02 CLD results. Protein concentration was determined using Protein A UPLC.

Protein expression and purity assays

MW and pI are often used to estimate the purity of antibody preparations[20]; however, the increased variety and complexity of Fc fusion glycosylations can greatly affect theoretical pI and MW calculations.[21,22] CE-SDS analysis of Fc02 before and after deglycosylation by enzymatic digestion revealed that native Fc02 exhibits significant deviation from the theoretical MW, whereas deglycosylation resulted in a more accurate approximation with a narrower peak profile (Fig. 7).



The increased variation observed in the intact form is suggestive of heavy glycosylation, so we examined glycan subtypes in more detail.

Figure 7.
Approximation of theoretical MW by CE-SDS varies as a function of glycosylation. [Fc02 theoretical MW = 97 kDa (dimer)].

Assessing Fc-fusion product by mass spectrometry

Fc02 product was evaluated for intact mass as detailed in Table 5a. Further investigations into glycosylation were performed using the methodology in Table 5b.

Intact mass analysis of Fc02 supports sequence integrity with observed masses agreeing well with expected mass (Table 6) while antennary type 2 glycans were the most predominant forms, as typically observed with mAbs (Table 7). Mannose, sialic acid, fucose, and galactose modifications (Fig. 8) have significant effects on IgG-based therapeutics so monitoring glycans is crucial.[22] Product quality assays such as mass spectrometry are needed during process development and manufacturing to avoid irreproducibility or misleading observations in upstream and downstream applications. Here, we show that FUJIFILM Diosynth Biotechnologies has the capability to conduct this type of analysis in flexible Fc-fusion process development programs.

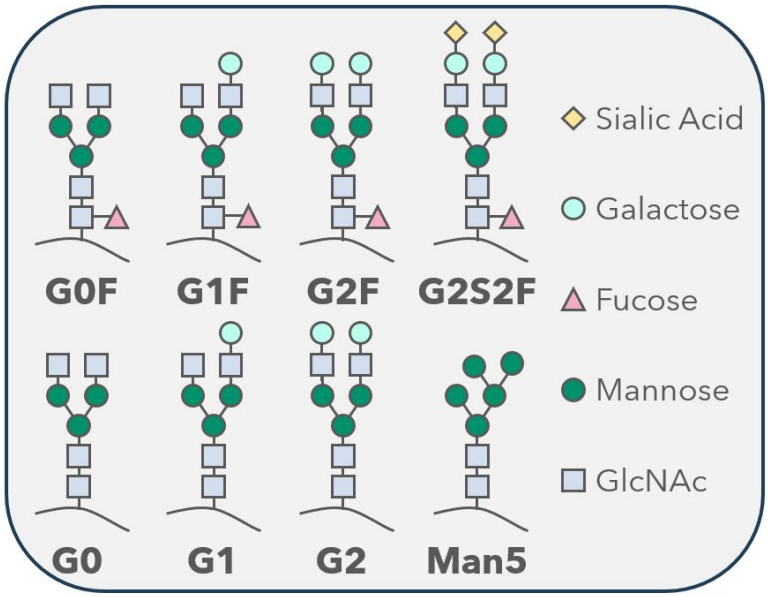


Figure 8. Common N-linked glycan types in mAb and Fc-fusion glycosylation.

Table 5a. Intact mass methodology.

BioAccord System:	Waters Acquity UPLC I-Class with TUV detector coupled to Acquity RDa Mass Spectrometer; Spectral data processed using Waters UNIFI software.
Analytical column:	Acquity UPLC Protein BEH C4, 300 Å, 1.7 µm, 2.1x50 mm
Mobile phase:	MPA: 0.1% Formic Acid in LC-MS UHQH_2O MPB: 0.1% Formic Acid in LC-MS Acetonitrile
Sample preparation:	Intact: Dilute to 1 mg/mL in UHQH_2O Deglycosylation: Diluted to 1 mg/mL treated overnight with PNGase F Reduced: Intact/deglycosylated sample treated with guanidine hydrochloride to denature and reducing agent
Sample loading:	2 µL
Flow rate:	0.2 mL/min
Run time:	10 minutes
Autosampler temperature:	6°C
Column temperature:	80°C
UV detection:	215 nm

Table 5b. Glycan analysis methodology.

BioAccord System:	Waters Acquity UPLC I-Class with fluorescence detector coupled to Acquity RDa Mass Spectrometer; Spectral data processed using Waters UNIFI software.
Analytical column:	Acquity Premier Glycan BEH Amide, 150 mm x 2.1 mm, 1.7 µm
Mobile phase:	Mobile Phase A: 50 mM Ammonium Formate pH 4.4 Mobile Phase B: Acetonitrile
Sample preparation:	Samples were prepared using the Waters RapiFluorMS standard protocol
Sample loading:	25 µL
Flow rate:	0.4 mL/min and 0.2 mL/min
Run time:	55 minutes
Autosampler temperature:	10°C
Column temperature:	60°C
Fluorescence detection:	Excitation=265 nm, Emission=425 nm

Table 6. Intact mass analysis of deglycosylated Fc02.

Protein name	Expected mass (Da)	Observed mass (Da)	Mass difference (Da)
Fc02	96897.2	96909.1	+11.9
Fc02 (reduced)	48458.7	48461.4	+2.7

Table7. Fc02 glycosylation analysis.

Species	% Total
Total Sialylated Glycans	11%
Mono Sialylated	9%
Di Sialylated	2%
Non-Sialylated Glycans	89%
Defucosylated Glycans	39%
Antennary 1 Glycans	10%
Antennary 2 Glycans	56%
Antennary 3 Glycans	3%
High Mannose Glycans	29%

Perspectives

Fc-fusion proteins have emerged as next generation biotherapeutics due to their improved pharmacokinetic properties and therapeutic activity, but despite intense investigation, development remains a challenge. The inherent complexity and structural diversity of these molecules can result in low expression, molecule instability, and inefficient processes. In this study, we evaluated the adaptability of the Apollo™ X mammalian expression system for expression of seven IGHG1-type Fc-fusion proteins of varying physicochemical characteristics.

Fed-batch culture of transfectant pools resulted in productivity levels ≥1 g/L for Fc-fusion proteins with a theoretical MW ≤ 110 kDa, a characteristic which describes most of the Fc-based therapeutics currently approved or in development. We believe that productivity of Fc-fusions with higher molecular weight may be improved by selecting clones with high Gene of Interest (GOI) mRNA expression or by taking measures to stabilize mRNA levels.

These data suggest that (i) mRNA transcript levels could be used to screen for cell lines with increased Fc-fusion productivity, and (ii) vector[23], cell line[24] and/or process optimization[25] strategies to increase mRNA abundance via increased transcription or mRNA stability. These strategies are options for future developments within FUJIFILM Diosynth Biotechnologies.

Having the ability to evaluate Fc-fusion product quality quickly and reliably is crucial to ensure safety and efficacy in downstream applications. Antibody preparation purity is often assessed via MW and pI estimations; however, these conventional methods can be rendered impractical given the extensive glycosylation of Fc-fusion proteins. Glycosylation has implications on Fc-fusion stability, pharmacokinetics, and immunogenicity, and must be closely monitored throughout biologic manufacturing.[26] Here, we confirm the effect of glycosylation on MW estimations, with CE-SDS indicating a significant shift in mass for deglycosylated product in comparison to intact product and mass spectrometry of deglycosylated product confirming expected mass. We propose an abbreviated workflow that allows for accurate and reliable evaluation of product homogeneity and quality.

The versatile Apollo™ X mammalian expression system combines cell line development, automated productivity screening, and manufacturability assessment services into one effective, integrated solution for monoclonal and bispecific antibodies, and Fc-fusion proteins. Our ability to adapt well established methods to assess product quality early in the process chain further streamlines Fc-fusion protein development, propelling these products through critical milestones with quality and patient safety at the fore.

Authors

Dr. Kalsi is a Principal Scientist in Mammalian Upstream Process Development,
Dr. Saunders is the Head of Mammalian Cell Culture,
Dr. Pybus is a Senior Staff Scientist in Mammalian Upstream Process Development,
Jessica Kane-Fidgeon is a Principal Scientist in Analytical Development,
Daniel Sharkey is a Research Scientist in Analytical Development,
Dr. Keen is a Senior Staff Scientist in Analytical Development
at FUJIFILM Diosynth Biotechnologies, Belasis Avenue, Billingham, TS23 1LH, UK.

Mr. Murakami is a Research Manager in Cell Science and Engineering,
Mr. Matsuura is a Senior Scientist in Mammalian Cell Line Development,
Mr. Seko is a Scientist in Mammalian Cell Line Development
at FUJIFILM Bio Science and Engineering Laboratories, Ashigarakami-gun, Kanagawa, Japan.

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