Accurate comparability assessment of a biosimilar interferon in process development

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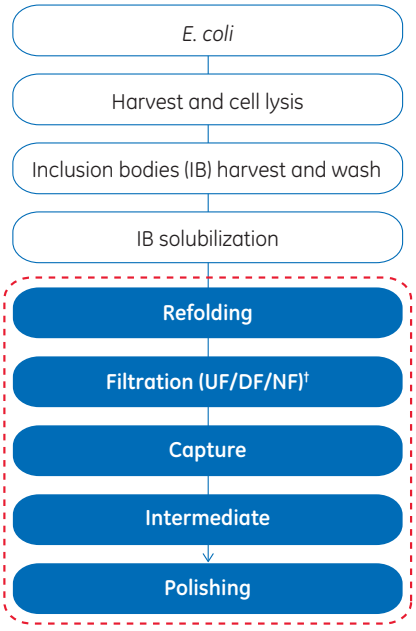
This application note describes how to achieve accurate comparability assessments using Biacore™ T200 and Amersham™ WB system in the development of a biosimilar. This is exemplified in this study by the use of these systems in the high-throughput process development (HTPD) of a biosimilar molecule, interferon α-2a (IFNα-2a)*. Biacore T200 was extensively used starting from the refolding step, through filtration procedures, followed by three chromatographic purification steps: capture, intermediate purification, and polishing (Fig 1). Two types of surface plasmon resonance (SPR) experiment made it possible to: (i) assess the comparability to the originator molecule; (ii) find the best refolding conditions; (iii) select the most appropriate chromatographic media (resins); and (iv) optimize the binding and elution steps. Amersham WB system was used to analyze the purity and confirm accurately the identity of the IFNα-2a biosimilar with fluorescence multiplexing detection.

Introduction

Biosimilars are considered to be one of the fastest growing sectors of the pharmaceutical industry. Since the launch of the first generation of biopharmaceutical products, manufactured using recombinant technologies in the 1980s, many new therapies for life-threatening and rare illnesses have been introduced. Years later, these products are now coming off patent and the introduction of biosimilars on the market is thus possible.

* Note that none of the GE Healthcare products described in this application note are therapeutic or diagnostic, but can be used as tools in drug research/discovery and the manufacture of biopharmaceuticals.



† Ultrafiltration/diafiltration/normal-flow filtration

Fig 1. Overview of the developed process for IFNα-2a. SPR analyses using Biacore T200 were used for steps indicated within the red rectangle. Western blotting using the fully integrated Amersham WB system was performed to confirm purity of the various chromatography steps as well as that of the recovered IFNα-2a.

The regulatory landscape is also changing, and regulations covering market approvals are evolving towards more open and abbreviated approval pathways. Developers of biosimilars use an increasing array of biomolecular characterization to convince regulators that their processes are well understood, robust, and controlled.

This is in line with the U.S. Food and Drug Administration (FDA) regulatory initiative Quality by Design (QbD), which requires that product quality goals are clear, process characterization is thorough, and risks are well mitigated.

Biosimilars are designed to be equivalent, both functionally and structurally, to the original (branded) biotherapeutic molecule (often referred to as the reference or originator product). One obstacle in producing a biosimilar is that the original process used to manufacture the reference molecule is unknown. To confirm the biosimilarity to the original reference product, extensive analytical studies including comparative physicochemical and functional studies are needed to confirm similarity. According to the FDA, the biosimilar and the originator "must be highly similar...., with no clinically meaningful differences in terms of safety, purity, or potency." The current focus towards a limited number of biosimilar targets means that the competition to be first to market is fierce, and once entering the market, the price of the drug is another area of competition. Therefore, the possibility to produce the drug at a competitive price is to a large extent determined by how the production process has been designed. Modern chromatography media and analytical methods such as SPR provide fast and reliable information on product quality and comparability in a process, increase productivity and flexibility, save time, and lower production costs.

This application note describes a process for HTPD of an IFNα-2a, which is compared throughout development with a reference molecule available on the market. The focus is on the use of Biacore T200 and Amersham WB system for accurate comparability studies during this development. Further details of the methods used in the production process are described in reference 1. Note that the intention of this work is not to produce a pharmaceutical product entering into clinical trials.

IFNα-2a expression, refolding, and purification

IFNα-2a is a molecule containing 165 amino acids with two disulfide bridges and with a calculated isoelectric point (pI) of 5.99. The protein was expressed as inclusion bodies (IB) in an *E. coli* system. After cell lysis and removal of cell debris by centrifugation, the IB was prepared by centrifugation. The IB fraction was washed and then solubilized in 8 M urea. From this step of process development, SPR analyses with Biacore T200 were included, starting with optimization of the refolding conditions (Fig 1).

The refolded interferon was transferred to a buffer suitable for the first chromatography step. Screening for suitable chromatography media was performed using either 1 ml HiTrap™ prepacked columns, chromatography media packed in Tricorn™ columns, or PreDictor™ 96-well filter plates.

Three chromatography steps were used, for which the media were selected using SPR analysis: capture with Capto™ S, intermediate purification step with Capto adhere ImpRes, and a polishing step operated in flowthrough mode using Capto Octyl. The overall yield of the process was 34%, while yield from the capture to the polishing step was 84%. Buffer exchange between the chromatography steps was not necessary, since only minor conductivity reduction by dilution with no buffer pH adjustments was used.

Two types of SPR assay using Biacore T200

Two types of SPR experiment were used, providing critical information at various steps throughout the process development. Since the SPR analyses are selective in terms of protein recognition, meaning that only the protein of interest (here IFNα-2a) is "visible", the experiments can be performed in the presence of impurities. Therefore, Biacore T200 is a good choice for the development of any purification procedure.

Concentration assay

Biacore T200 was used to determine active concentration of refolded and denatured IFNα-2a. The amount of protein was assessed using Calibration-Free Concentration Analysis (CFCA), by measuring binding to two conformation-specific antibodies recognizing the folded and unfolded IFNα-2a form, respectively (Fig 2A). Anti-IFNα-2a antibodies with specificity against denatured and folded interferon were covalently coupled to Sensor Chip CM5 using a standard amine coupling procedure. Concentration (by CFCA) of commercially available originator molecule was in agreement with the specification of supplier, and was measured at the beginning and the end of each experiment to ensure the reliability of all concentrations determined.

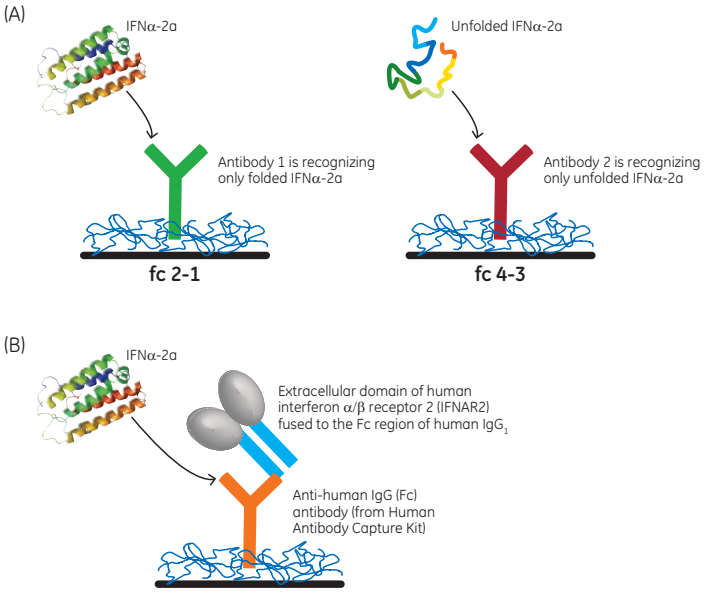


Fig 2. SPR assay setup used in the process development of IFNα-2a. (A) Simultaneous CFCA of folded and unfolded fraction; (B) binding kinetics to human interferon α/β receptor 2 (IFNAR2).

CFCA is based on the measurement of initial binding rates at two different flow rates under certain conditions of mass transport limitation (2, 3), and does not require the use of an internal standard. A sample is injected twice during ~ 30 s at two flow rates, typically 5 and 100 µl/min. Concentration is calculated by fitting the binding curves obtained at the two flow rates to a 1:1 binding model (4).

Kinetic analysis

The comparability to originator molecule was assessed by measuring binding kinetics to interferon α/β receptor 2 (IFNAR2) at each step of process development (Fig 1 and 8). IFNAR2 used in this study is a chimeric protein encompassing an extracellular domain of IFNAR2 fused to the Fc region of human IgG_1. This construct could be easily captured on anti-human IgG (Fc) antibody (from Human Antibody Capture Kit, GE Healthcare), which was covalently coupled to Sensor Chip CM5 (Fig 2B). The kinetics of interferon-to-receptor binding was then assessed by injecting a concentration series of 0.6 to 10 nM IFNα-2a over the surface.

Optimization of refolding step

CFCA was used to quickly find the best refolding conditions by measuring binding of folded and unfolded form in parallel and calculating yield of refolding from concentration of folded fraction in relation to total concentration (folded and unfolded; Fig 2A). The two antibodies, recognizing the refolded and denatured IFNα-2a forms, were immobilized in flow cell 2 and 4, respectively, enabling in-line monitoring of refolding. A typical single-concentration measurement was taken in ~ 2 min. Sample depletion from flow cell 2 to 4, determined in a separate experiment, was about 10%, and concentrations determined in flow cell 4 were corrected with this factor.

Conditions for refolding were studied using a Design of Experiment (DoE) approach, looking at pH (range 8.0 to 10.0), arginine concentration (range 0 to 0.5 M), dilution (10- and 50-fold), as well as addition of a glutathione redox system (GssG/GssH). The highest yield in the refolding step was achieved when the denatured IFNα-2a was diluted in 8 M urea to a final concentration of 0.5 to 0.6 mg/ml before refolding. The degree of dilution and the use of a GssG/GssH system did not show a significant effect. The optimal buffer for refolding was found to be 50 mM Tris at pH 10 containing 0.5 M arginine, which gave 64% refolding yield (Fig 3).

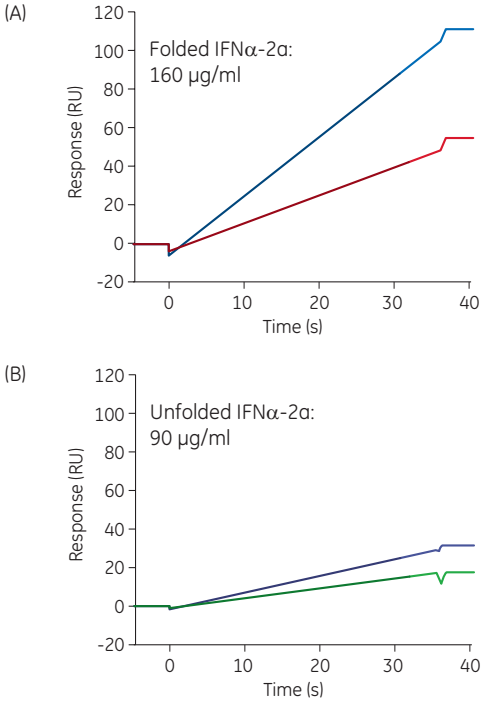


Fig 3. Typical CFCA sensorgrams showing sample injected at 5 and 100 µl/min through all flow cells, with antibodies recognizing folded and unfolded IFNα-2a immobilized in flow cells 2 (sensogram A) and 4 (sensogram B), respectively. Flow cells 1 and 3 were used as unmodified references for flow cells 2 and 4, respectively. Measured concentrations multiplied by a dilution factor of 1000 are shown in corresponding sensorgram windows.

Selection of the most appropriate chromatographic media

CFCA was used to select the most appropriate media in three chromatographic purification steps: capture, intermediate purification, and polishing (Fig 1). The IFNα-2a concentrations were measured in fractions applied to and eluted from either PreDictor 96-well plates, 1 ml HiTrap prepacked columns, or Tricorn columns packed with media (runs being performed on ÄKTA™ avant 25 system). All fractions were diluted 1000- to 10 000-fold prior to CFCA. Media were screened for dynamic binding capacity and recovery. Fraction bound and recovery were calculated with respect to amount of protein applied to the plates or columns.

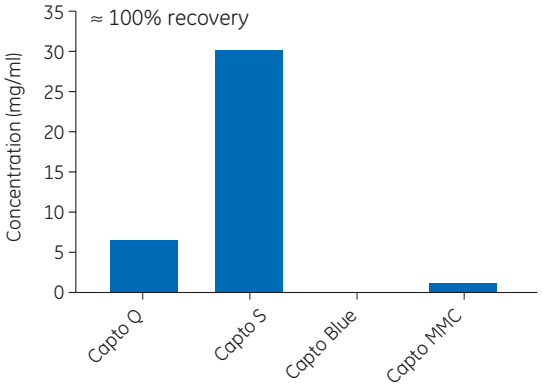


Fig 4. Comparing the suitability of four chromatography media—Capto Q, Capto S, Capto Blue, and Capto MMC—for the capture step by CFCA of eluted IFNα-2a. Recovery for Capto S, shown in the plot, was calculated in relation to applied amount of protein.

Media screened for the capture step were: the anion exchangers Capto Q and Capto adhere; the cation exchangers Capto S, and Capto MMC; the affinity medium Capto Blue; and the hydrophobic interaction chromatography (HIC) media Capto Butyl and Capto Octyl. Anion exchangers, affinity, and HIC media were operated at high pH and cation exchangers at low pH. Among the screened media, Capto S provided optimal results with ~ 30 mg/ml protein in eluted fractions and ~ 100% recovery (Fig 4).

Optimization of binding and elution conditions for intermediate purification

The optimization of binding and elution conditions for the intermediate step was performed by CFCA. Binding conditions were screened by measuring the concentration of start material and flowthrough fractions from PreDictor 96-well plates filled with three media—Capto MMC ImpRes, Capto adhere ImpRes, and Capto Q ImpRes—rinsed with buffers with various composition. Forty-eight buffer conditions were analyzed for Capto MMC ImpRes and Capto adhere ImpRes, and 32 buffer conditions were tested for Capto Q ImpRes by varying salt concentration from 0 to 700 mM NaCl and pH from 5.0 to 9.0. One unattended overnight experiment was needed for each medium tested. Fraction bound was calculated from the difference between start and flowthrough concentration. Capto MMC ImpRes showed a binding of > 80 mg INFα-2a/ml medium at pH 5.0 and 0 M NaCl (Fig 5). Capto adhere ImpRes showed a binding of > 80 mg INFα-2a/ml medium at pH 7.0 and 0 M NaCl and a broader range of binding than the other media tested. Capto Q ImpRes showed the lowest binding capacity of the three media tested and was eliminated from selection as a medium for the intermediate purification step.

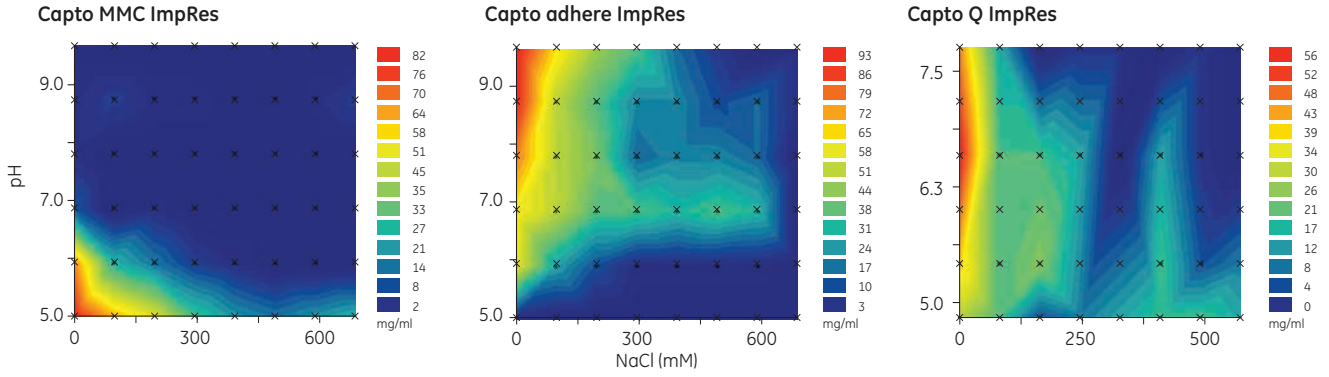


Fig 5. Selection of the most appropriate chromatography medium using a DoE approach. Capto MMC ImpRes, Capto adhere ImpRes, and Capto Q ImpRes (in PreDictor 96-well plates) were tested for optimization of binding conditions (by varying buffer composition: salt concentration from 0 to 700 mM NaCl and pH from 5.0 to 9.0) for the intermediate purification step. Fraction bound, calculated from the difference between concentrations of start and flowthrough fractions, was used as response. Results obtained from analysis by Assist software are shown as contour plots for each medium.

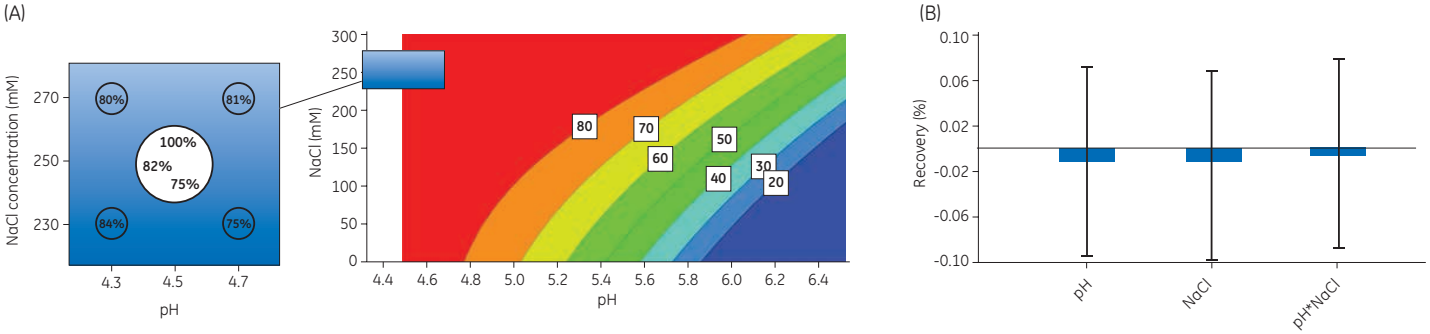


Fig 6. Optimization of elution step on Capto adhere ImpRes using DoE. (A) Results from CFCA of fractions eluted from prepacked or packed chromatography columns using buffers of varying NaCl concentration (0 to 300 mM) and pH (4.5 to 6.5) are visualized in contour plot by MODDE software (Umetrics). Small variations in buffer composition in a robustness study, indicated by the blue inset, resulted in 75% to 100% recovery. (B) The coefficient plot for the robustness study with recovery of IFNα-2a as response. Regression coefficients are insignificant and crossing zero indicating that the elution step is robust to changes in pH and NaCl concentration.

Capto adhere ImpRes was selected for the intermediate step due to the broadest binding capacity range and highest purity (data not shown).

Using 1 ml HiTrap prepacked columns and Tricorn columns packed with media, the elution conditions from Capto adhere ImpRes were optimized. A DoE study, looking at pH (4.5 to 6.5) and salt concentration (0 to 300 mM NaCl) was run, using a Central Composite Face (CCF) design with 11 experiments (three center points). Concentration analyses of fractions eluted found the optimal elution conditions, giving the highest concentration of eluted IFNα-2a, at low pH and salt concentration of 150 to 300 mM NaCl (Fig 6A).

In a subsequent robustness study, a Full Factorial (2 levels) orthogonal balanced design was applied. After equilibrating the column with 50 mM sodium phosphate pH 7.8, a step gradient elution was performed, varying the salt concentration from 230 to 270 mM NaCl and pH from 4.3 to 4.7, with concentration of eluate as response. The small variation of NaCl concentration and pH resulted in 75% to 100% recovery, calculated as the ratio of eluted-to-applied concentration (Fig 6A). The elution step was robust to changes in pH and NaCl concentration since the confidence intervals of regression coefficients were all crossing zero, meaning that the coefficients were not significant (Fig 6B).

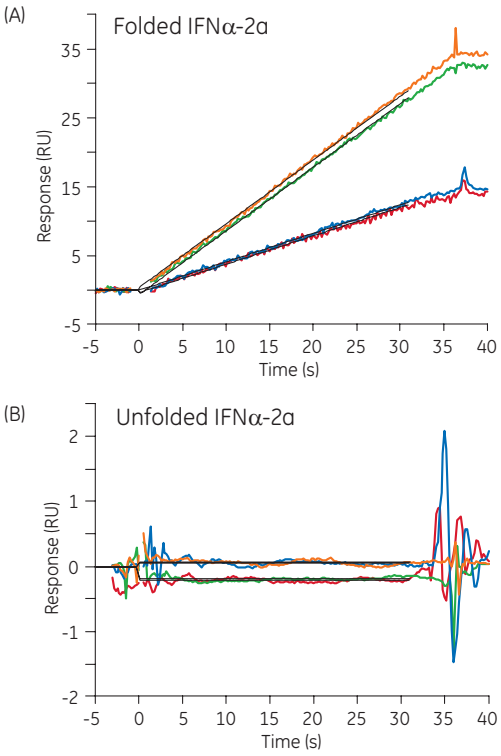


Fig 7. Sensorgrams showing CFCA of the final product obtained from HIC using Capto Octyl. The sensorgrams show curves obtained from concentration measurement on antibodies recognizing (A) folded and (B) unfolded IFNα-2a, respectively. Concentration of folded protein was 0.7 mg/ml (dilution prior to CFCA was 1000-fold). For the analysis of unfolded fraction, the protein was not diluted, and no response was observed

Selection of media for polishing step

IFNα-2a was at a high level of purity already after the second, intermediate purification step, but as a precautionary measure a third, polishing step was introduced. The choice of chromatography technique for polishing stood between preparative size exclusion chromatography, SEC (also called gel filtration) and HIC. SEC was excluded due to its low productivity (results not shown). Several HIC media were screened and the chosen medium was Capto Octyl, which could be run in flowthrough mode using a gradient from 0% to 30% of isopropanol to elute the protein. The concentration of the final product was 0.7 mg/ml and recovery ~ 100%, with no unfolded protein detected (Fig 7).

Assessment of comparability to originator molecule

The concentrations of folded IFNα-2a, obtained from CFCA at each step of process development, were used to prepare the concentration series (from 0.6 to 10 nM) for the interaction study with surface-attached IFNAR2 (Fig 2B). It was crucial to know the concentration of folded, capable of binding IFNα-2a for accurate kinetic characterization. Traditional methods, such as UV absorption or colorimetry, determine the total protein concentration that can be higher than the concentration of protein capable of binding. The association rate constant, k_a, is connected to protein concentration. When this concentration is overestimated, then k_a is underestimated and dissociation equilibrium constant, K_D (calculated as a ratio k_d/k_a), is overestimated. This gives seemingly lower affinity, which can lead to incorrect conclusions about comparability to the originator molecule. The major advantage of using CFCA was that knowing the binding concentration of IFNα-2a, the binding kinetics to IFNAR2 could be monitored in the presence of nonbinding unfolded fraction, host cell proteins, and other impurities. Therefore, very early in the process development, it was possible to ensure comparability in terms of kinetic and affinity constants. Rate constants were calculated from replicate experiments by globally fitting the binding curves to a 1:1 binding model (Fig 8). Varied surface densities of IFNAR2 were used to improve the robustness of the evaluation. The k_a and k_d values were within 2 × standard deviation (SD) of the mean value for originator molecule and affinity was ~ 2 nM (Fig 8).

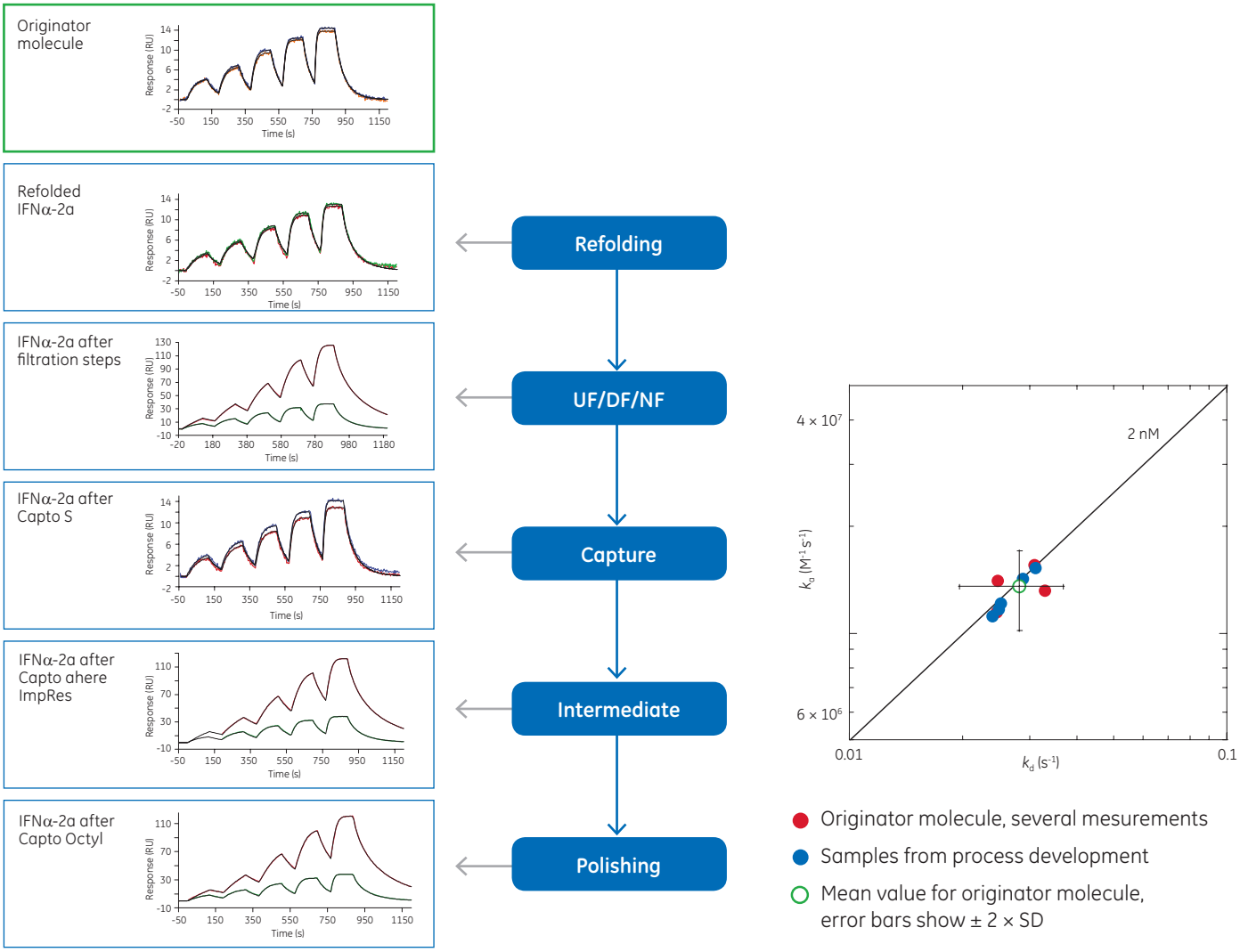
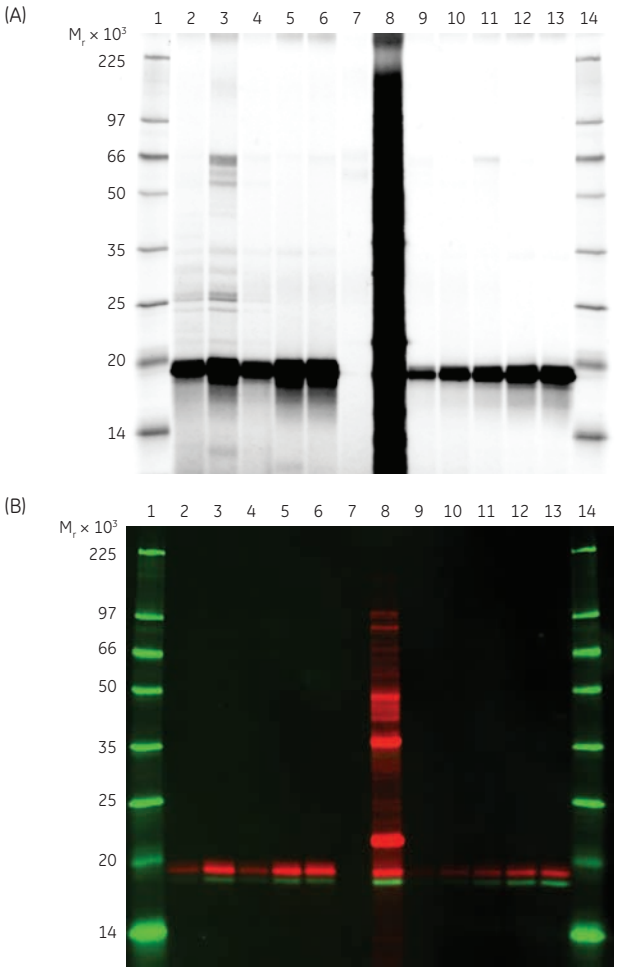


Fig 8. Replicate sensorgrams (concentration series of 0.6 to 10 nM) from surfaces with varied IFNAR2 density (red and green curves) are shown to the left. Corresponding steps in the process development are shown to the right. The sensorgram for the originator molecule is shown at the top. Data was globally fitted to the 1:1 binding model (overlaid black fitted curves). The k_a/k_d chart, to the right, shows that the rate constants for all samples from the biosimilar development are < 2 × SD from the mean value for originator molecule.

Purity analysis and identity confirmation with Amersham WB system

To assess the purity in different purification steps and to confirm the presence and identity of IFNα-2a, a Western blot experiment was run using the Amersham WB system. The system is a fully integrated Western blotting platform where every stage of the Western blotting process—electrophoresis, scanning, transfer, and probing—is standardized and monitored. This approach minimizes the assay variability inherent with Western blotting to provide quantitative data with fewer repeats and control experiments using fluorescence multiplexing.

The different sample fractions were prelabeled with ready-to-use consumables including Cy™5 dye, labeling buffer, and loading buffer and using a quick protocol with incubation at 95˚C for 5 min. After prelabeling, samples were loaded on an Amersham WB gel card 13.5%. Electrophoresis was run at 50 mA for 5 min and then paused for in-well sample clean up using Amersham WB paper comb. Electrophoresis was restarted and run for approximately 42 min total, after which it was automatically stopped as the dye front was detected to be exiting the gel. After electrophoresis, the gel card was automatically scanned for Cy5 providing an image of total protein signals for purity analysis. The gel card was then removed and prepared for transfer onto an Amersham WB PVDF card. Transfer was run for 30 min at 100 V. The PVDF card was removed and transferred to the probing unit and processed for 2.5 h with primary antibodies against IFN α-2a and Amersham WB goat anti-mouse Cy3 secondary antibodies. After probing, the PVDF card was dried and scanned for Cy5 and Cy3 signals using the integrated laser scanner in the Amersham WB system. The resulting multiplexed image shows both the total protein content (Cy5) and the specific antibody signal (Cy3) for IFNα-2a (Fig 9).



Lanes

1 and 14.	Amersham WB molecular weight markers.
2.	UF/DF/NF start material for Capto S.
3.	Capto S eluate + NaCl.
4.	Capto S eluate pH + NaCl.
5.	Capto adhere eluate (DBC).
6.	Capto adhere eluate (pool).
7.	Capto adhere eluate fraction 11.
8.	IFNα-2a culture.
9 to 13.	originator molecule reference (dilution series).

Fig 9. Purity analysis and identity confirmation using Amersham WB system. (A) Gel card image shown with high-contrast settings. Cy5 signals represent the total protein content in each sample. Calculating the amount of IFNα-2a target signal compared to the total signal gives purity levels at 99.4% for the Capto adhere ImpRes intermediate fractions. (B) Western blot membrane card. The multiplex image shows the signal representing the total protein content in red (Cy5) and the specific antibody signal against IFNα-2a in green (Cy3). A slight shift can be seen between the two signals where the Cy5 dye adds size to the IFNα-2a molecule. This is expected for low molecular weight samples.

Conclusions

The interferon biosimilar purification process was developed using a combination of concentration analyses with Biacore T200, DoE to optimize the purification steps, and Amersham WB system for purity confirmation.

Concentration assays using SPR provided by Biacore T200 could be easily adopted to DoE format and quickly provided information about amount of folded and unfolded protein in high-throughput, unattended experiments. Moreover, since the SPR assays are based on the detection of specific binding, a correct concentration of protein could be measured even in the presence of impurities. This selective mode of concentration measurement was needed to assess comparability of the biosimilar to the originator product early in process development.

DoE was used not only for the chromatography steps but also for the refolding step, a notoriously difficult step in the production of proteins that form IB when produced in *E. coli*. The amount of produced IFNα-2a biosimilar was used as a response to optimize all described steps by DoE.

Finally, purity of the various chromatography steps and positive identification of the IFNα-2a biosimilar was accurately and reliably confirmed by Western blotting with the integrated Amersham WB system. The target signals were calculated using fluorescence multiplexing.

References

1. Application note: Development of a production process for biosimilar interferon α-2a, GE Healthcare, 29-1338-22, Edition AA (2014).
2. Christensen, L. H. Theoretical analysis of protein concentration determination using biosensor technology under conditions of partial mass transport limitation. *Anal. Biochem.* **249**, 153–164 (1997).
3. Sigmundsson, K. *et al.* Determination of active concentrations and association and dissociation rate constants of interacting biomolecules: an analytical solution to the theory for kinetic and mass transport limitations in biosensor technology and its experimental verification. *Biochem.* **41**, 8263–8276 (2002).
4. Biacore T200 software handbook, GE Healthcare, 28-0500-11, Edition AA (2013).

Ordering information

Product	Code number
Biacore T200 Processing Unit	28-9750-01
Series S Sensor Chip CM5, pack of 3	BR100530
Human Antibody Capture Kit	BR100839
Amersham WB analyzer, including electrophoresis & scanning unit, transfer unit, and probing unit	29-0320-30
Amersham WB gel card 13.5%	29-0225-64
Amersham WB PVDF card	29-0225-66
Amersham WB paper comb	29-0562-86
Amersham WB goat anti-mouse Cy3	29-0382-75
Amersham WB molecular weight markers	29-0307-35
ÄKTA avant 25 system	28-9308-42
Capto Q ImpRes, 25 ml	17-5470-10
Capto adhere ImpRes, 25 ml	17-3715-01
Capto MMC ImpRes, 25 ml	17-3716-01
Capto Q ImpRes, 25 ml	17-5470-10
Capto S, 25 ml	17-5441-10
Capto Octyl	17-5465-00
PreDictor 96-well filter plates	Various, see Web site
HiTrap Capto adhere ImpRes, 1 ml	17-3715-10
Tricorn 5/100 empty column	28-4064-10

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