A Case Study on Comparability of a Monoclonal Antibody During Early Stage Development

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  - Binding to Fcγ-receptors using Biacore
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Introduction

- For phase II - III clinical development changes in drug substance manufacturing process were implemented:
  - production cell line (closely related)
  - manufacturing site
  - scale-up
  - drug substance formulation

- Two representative drug substance batches from old and new process were chosen for the comparability study to assess
  - the purification process
  - physicochemical property
  - stability (6 months)

Purification Process Performance

- The performance of the old and the new purification process was assessed by their capability to remove representative process-related impurities
- Host cell proteins, DNA, and column-leached Protein A were removed to a level below limit of quantitation in both processes
- The overall removal factors were comparable

Purification Process Performance

	Old Process		New Process	
	batch #1	batch #2	batch #1	batch #2
Host Cell Proteins (HCP) in ng/mg				
DSP-step 1	**$5 \cdot 10^5$**	**$2 \cdot 10^5$**	**$3 \cdot 10^6$**	**$1 \cdot 10^6$**
Drug Substance	**< 5**	**< 5**	**< 0.5**	**< 0.5**
Removal factor	**$>10 \cdot 10^4$**	**$>4 \cdot 10^4$**	**$>500 \cdot 10^4$**	**$>240 \cdot 10^4$**
DNA in pg/mg				
DSP-step 1	**$4 \cdot 10^7$**	**$2 \cdot 10^7$**	**$0.4 \cdot 10^6$**	**$1 \cdot 10^6$**
Drug Substance	**< 1.0**	**< 1.0**	**< 1.2**	**< 1.2**
Removal factor	**$>4 \cdot 10^7$**	**$>2 \cdot 10^7$**	**$>3 \cdot 10^6$**	**$>0.8 \cdot 10^6$**
Column-leached *Protein A* in ng/mg				
DSP-step 3	**1.6**	**7.4**	**7.0**	**8.1**
Drug Substance	**< 0.6**	**< 0.6**	**< 0.6**	**< 0.6**
Removal factor	**>25**	**>12**	**>11**	**>13**

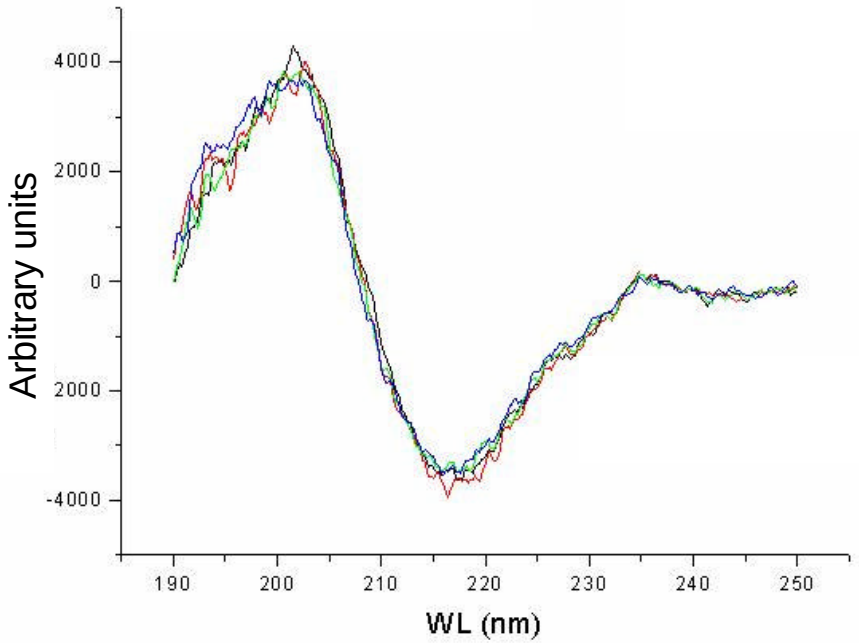
Conformational Changes by CD

- Conformational changes are checked by circular dichroism (CD) spectroscopy
- Far-UV Spectrum → sensitive to secondary structural features
  - relative abundance of a-helix, ß-sheets or random structures
- Near-UV Spectrum → sensitive to tertiary structure changes
  - reflects the environment around aromatic side chains
- Same antibody concentrations (10 mg/mL) and (mixed) buffer composition

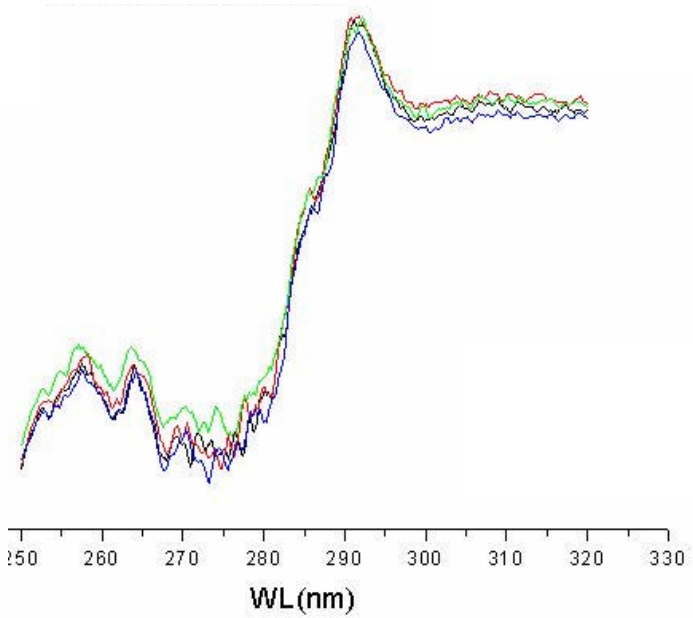
Conformational Changes by CD

Overlays of mean CD spectra for the four batches tested

Far UV CD



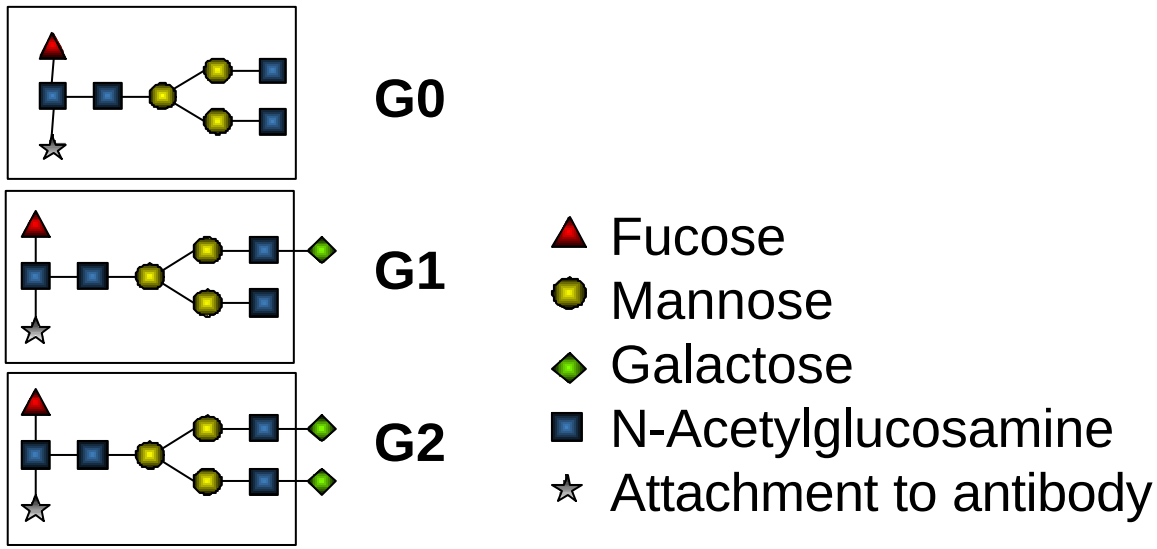
Near UV CD



All spectra are indistinguishable from each other indicating comparable antibody conformation

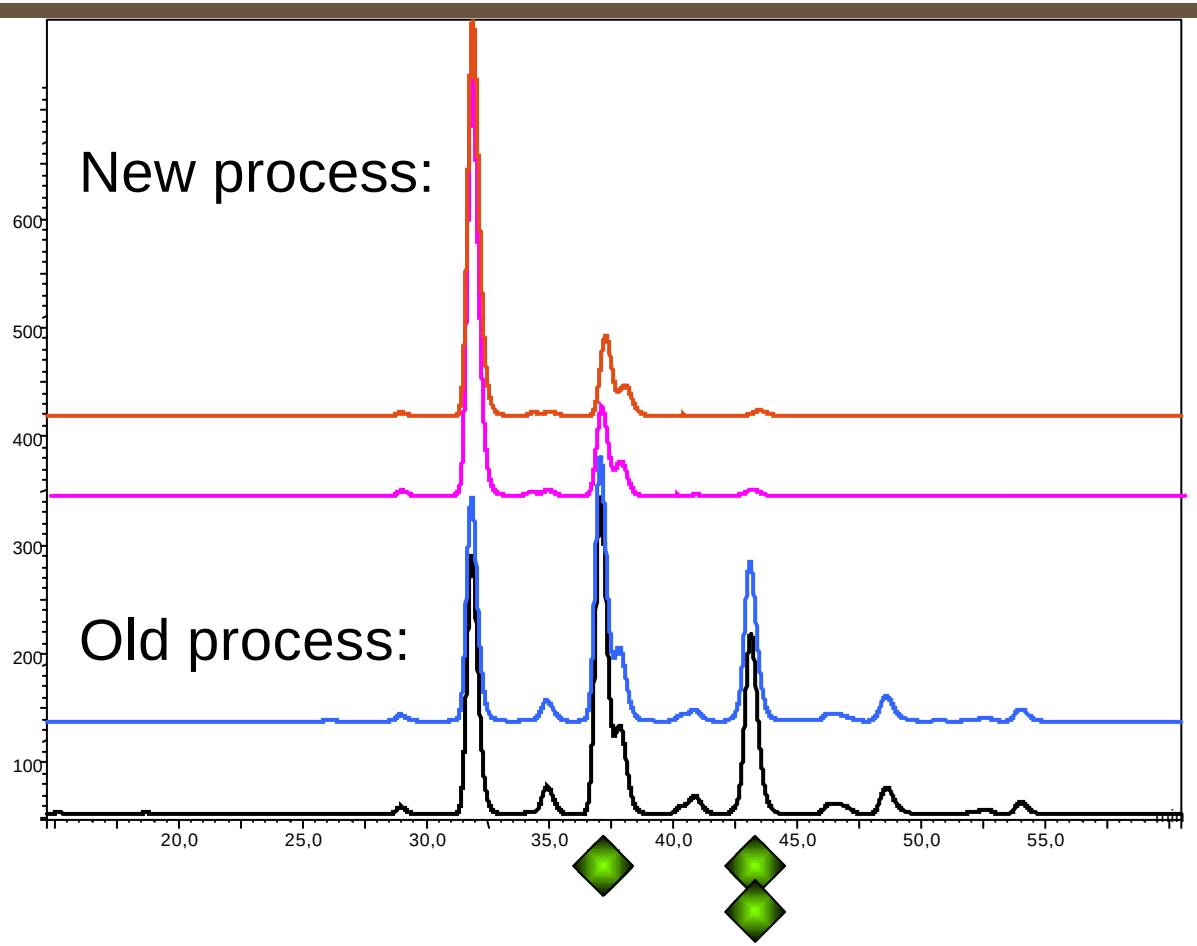
Glycosylation Profile

- Each of the heavy chains has one N-glycosylation site
- The oligosaccharides were quantified by HPLC after enzymatic cleavage and chemical derivatization (2-AB)
- The identity of the oligosaccharides was confirmed by mass spectrometry (data not shown)



Glycosylation Profile

Differences in glycan distribution were observed as expected after the cell line switch.



Antibody from new process has lower content of oligosaccharides with terminal galactose

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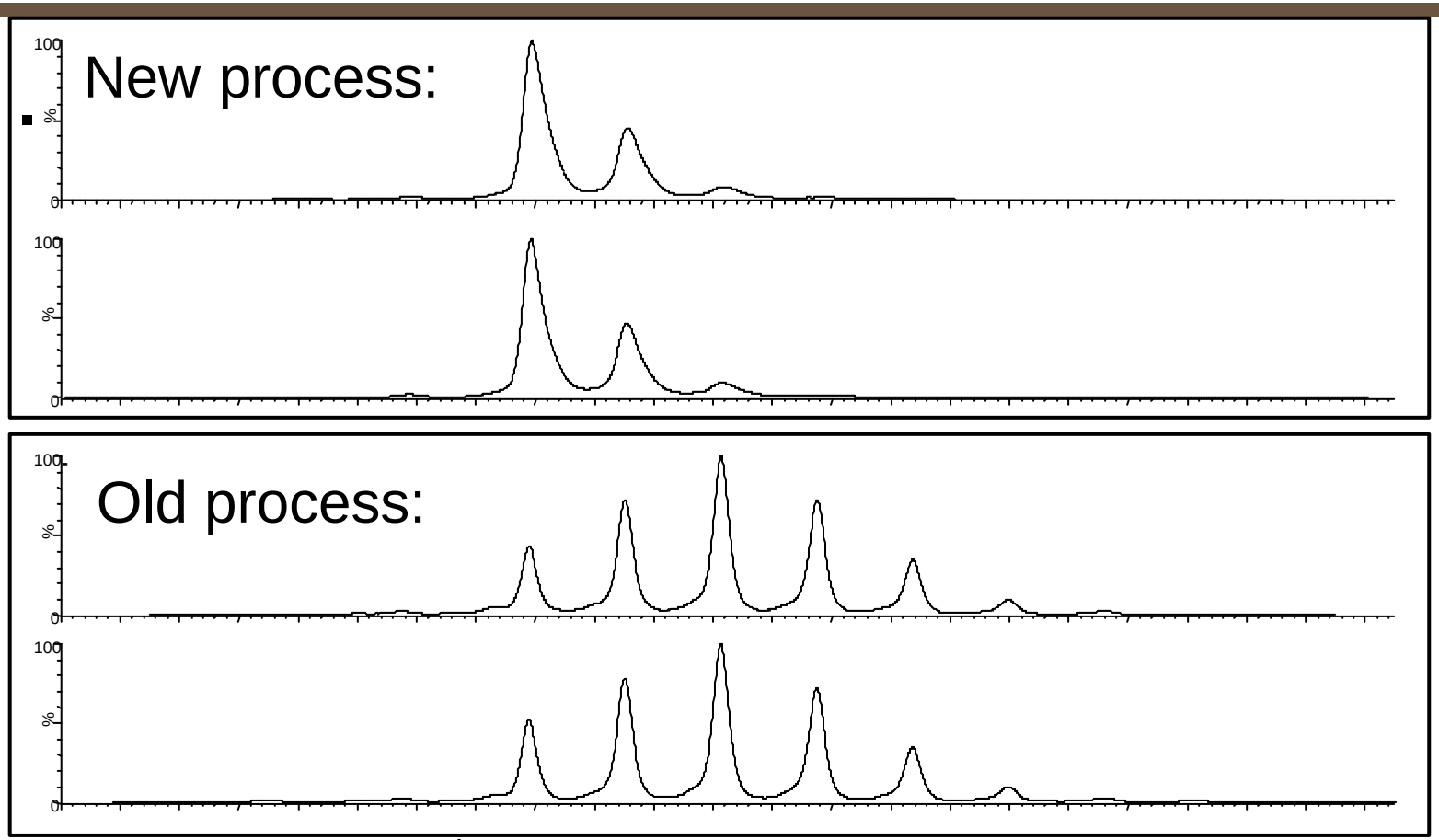
Molecular Mass of the Antibody

- The molecular masses of the antibody batches were determined using a Q-TOF mass spectrometer

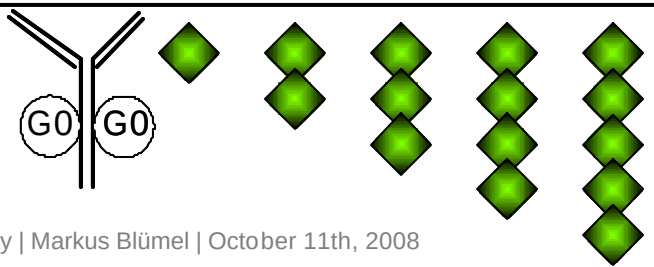
- Heterogeneity due to post-translational modifications at the heavy chains were removed:
  - removal of C-terminal lysines with Carboxypeptidase B
  - removal of oligosaccharides with PNGase F
  - removal of both C-terminal lysines and oligosaccharides

- The observed mass for each main component is within ± 6 Da of the expected mass.

Molecular Mass of the Antibody - after Removal of C-terminal Lysines



Heterogeneity by Glycosylation:



The expected masses were observed

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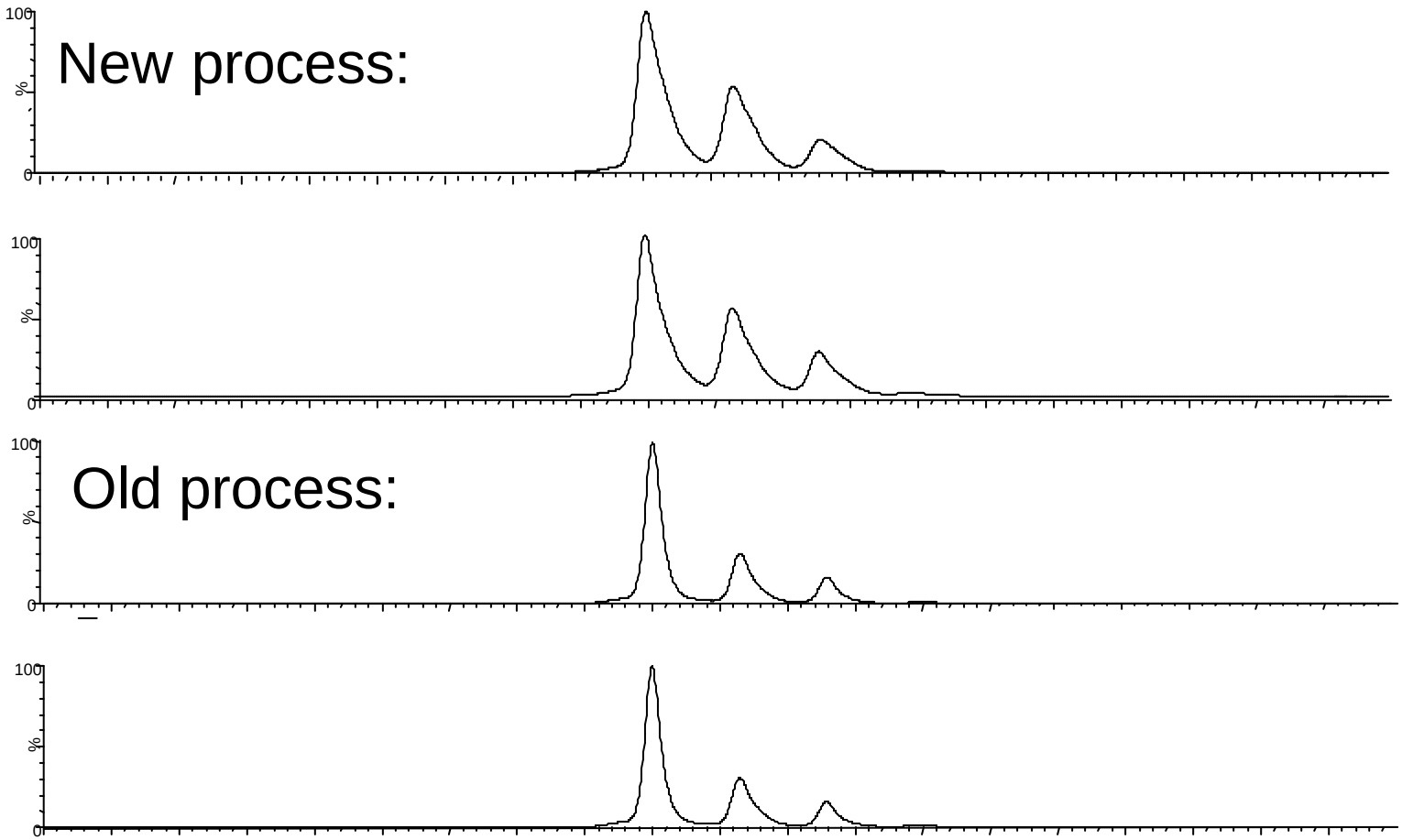
Molecular Mass of the Antibody - after Removal of Oligosaccharides



C-terminal lysine of heavy chain not completely removed
The expected masses were observed

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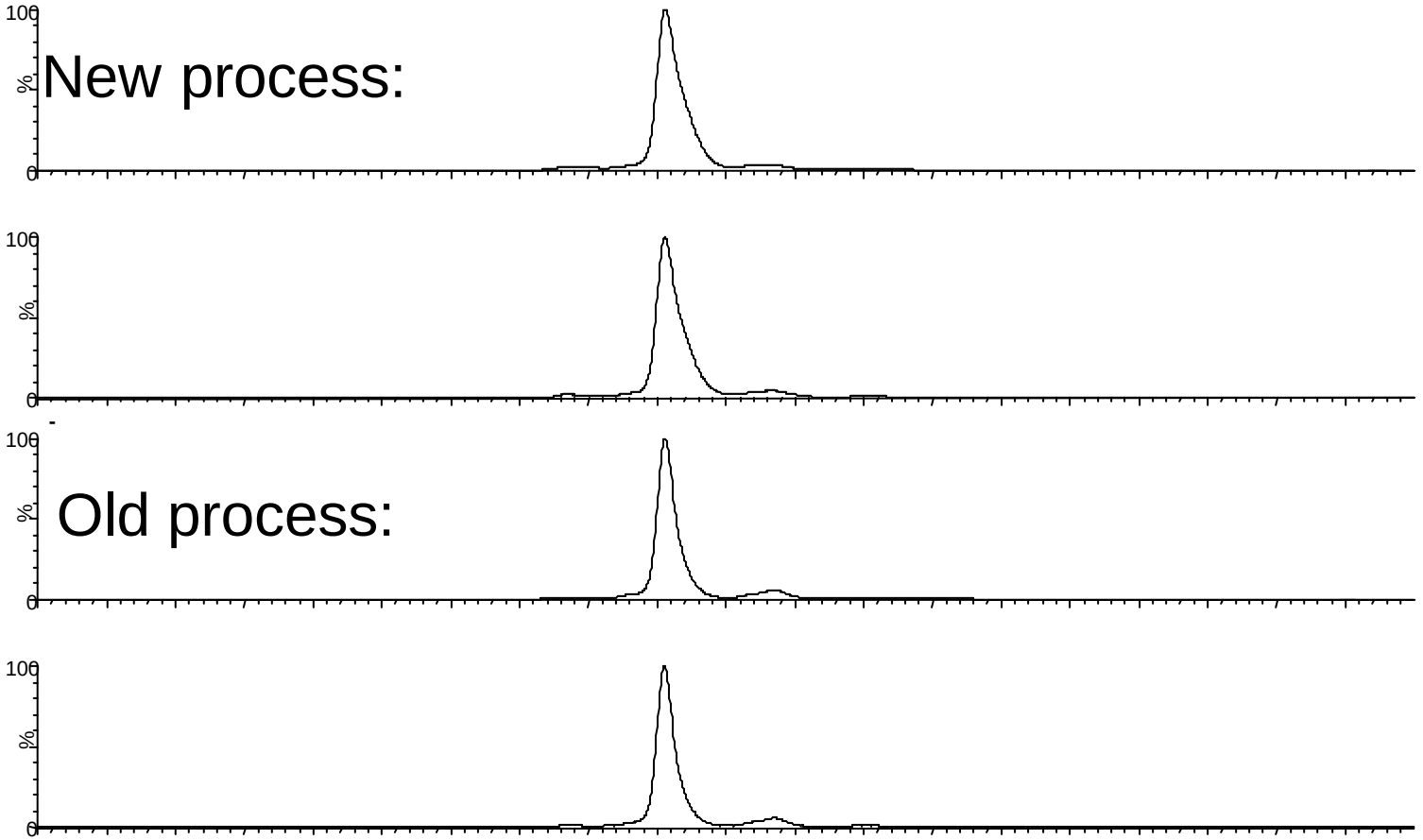
Molecular Mass of Antibody after Removal of C-terminal Lysines and Oligosaccharides



The expected mass was observed

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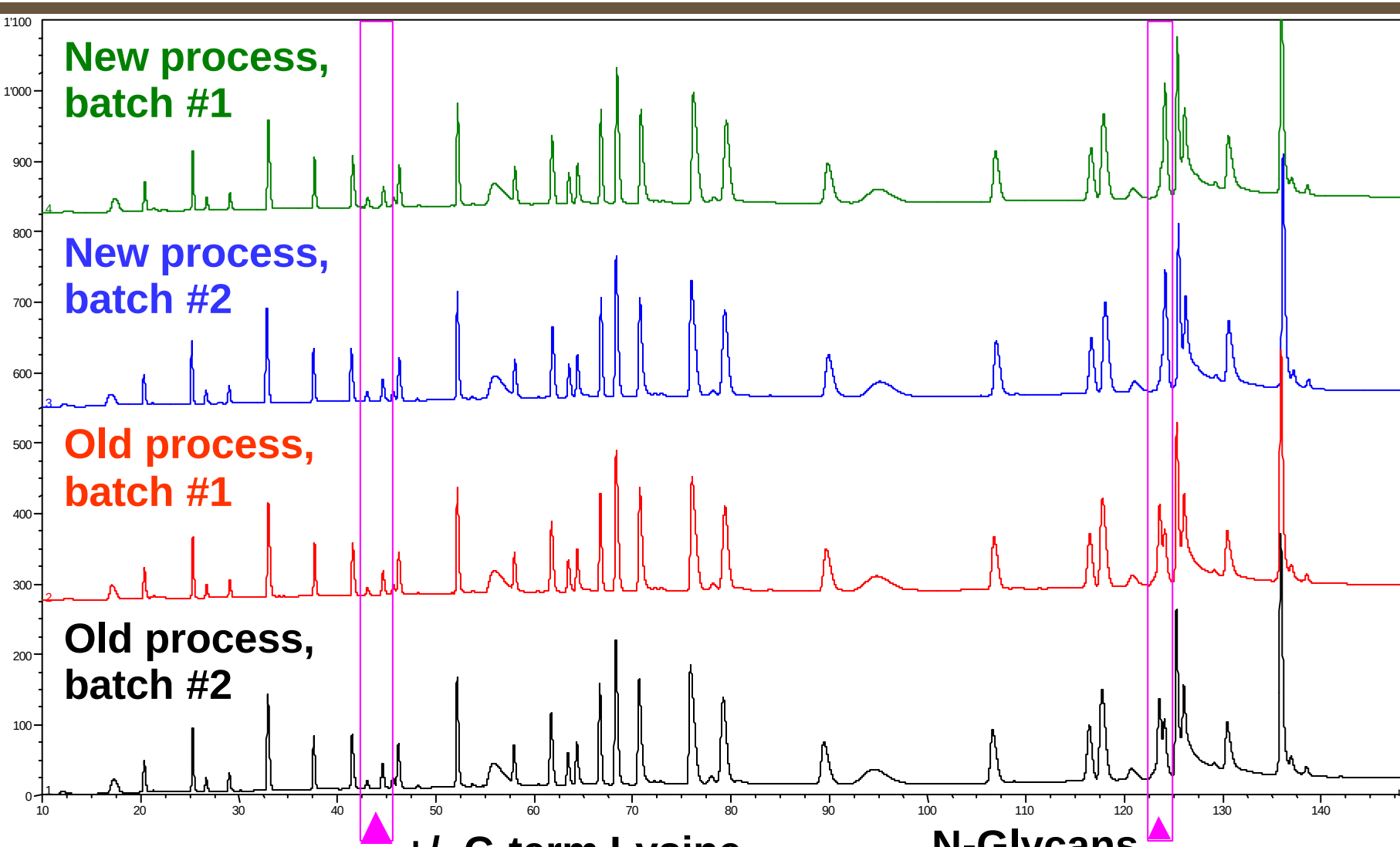
Identity by Peptide Mapping

- The identity of the antibody manufactured by the two processes was confirmed by peptide mapping
- The samples were denatured, reduced, alkylated and digested with endoproteinase Lys-C
- The peptides obtained after the digestion were resolved using reversed phase chromatography and identified by online electrospray ionization mass spectrometry
- The identity of each tested sample was confirmed by comparing the masses of the peptides identified with the theoretical masses

Identity by Peptide Mapping



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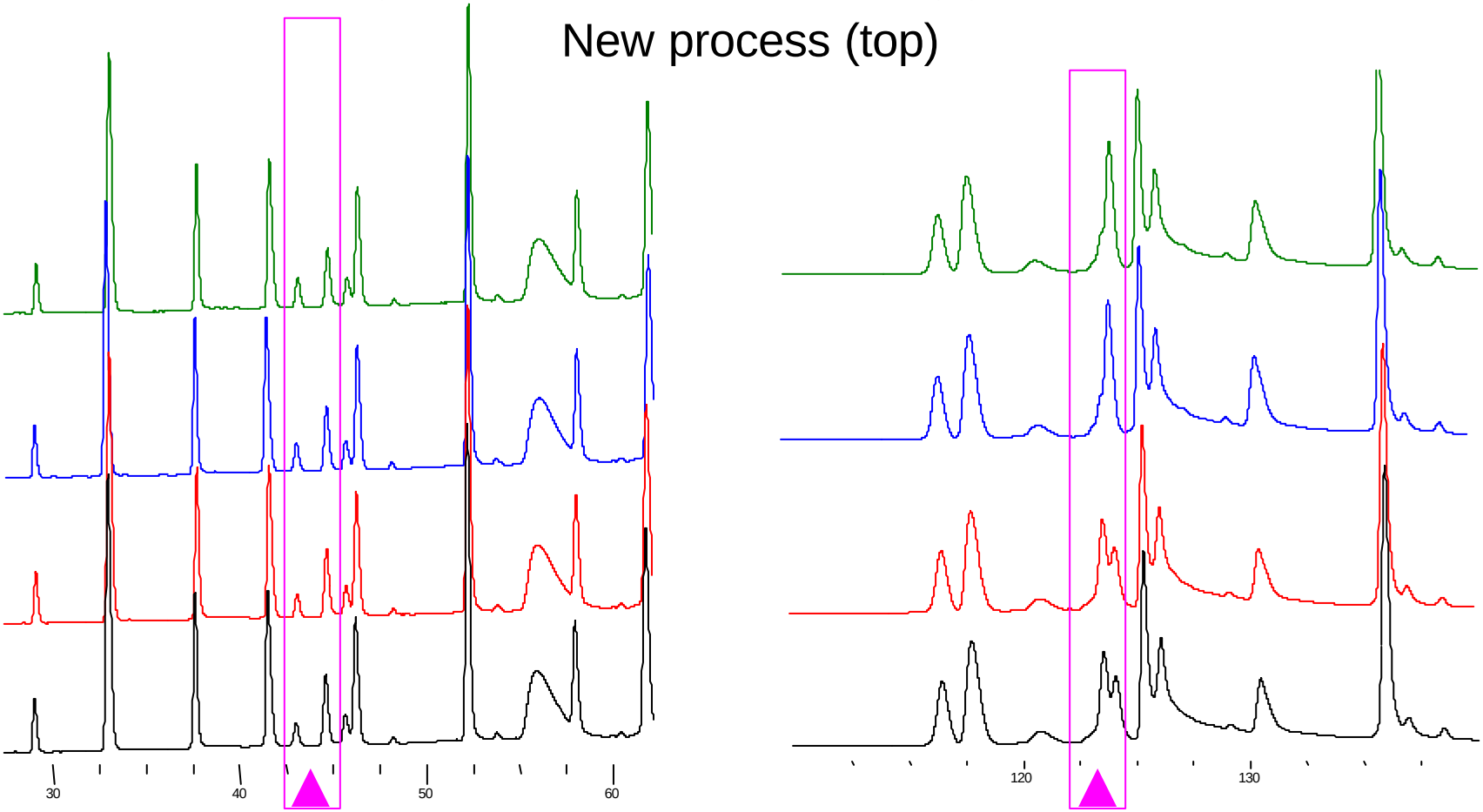
Identity by Peptide Mapping (zoom)

+/- C-terminal lysine

N-glycans

New process (top)



Old process (bottom)

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Identity by Peptide Mapping

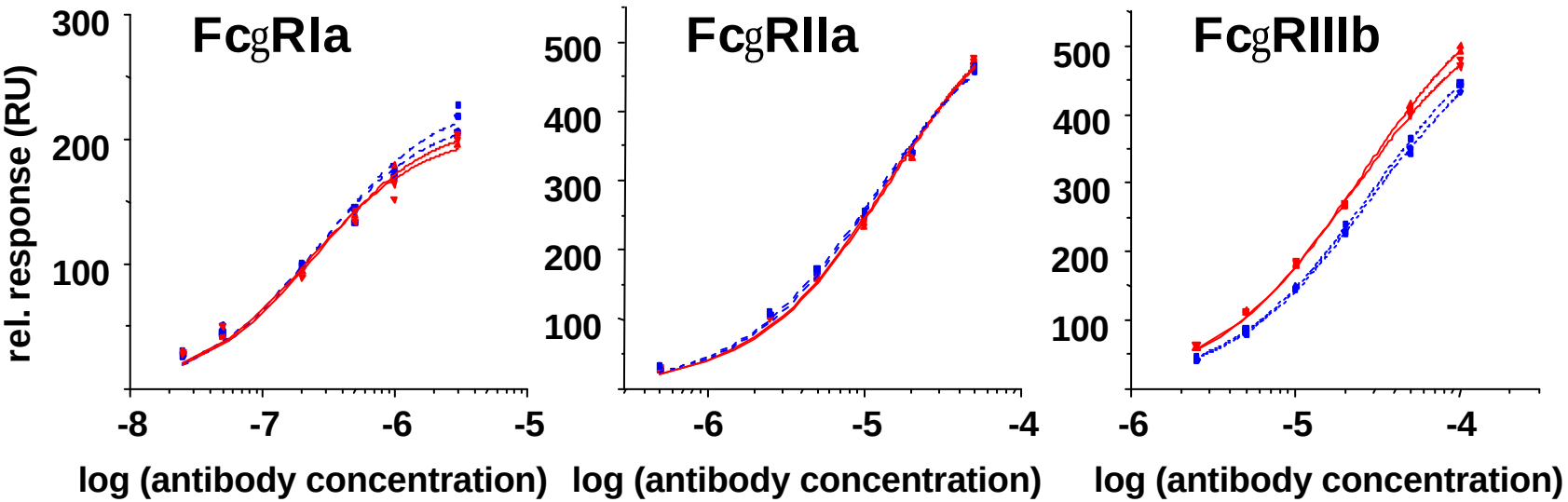
- The peptides identified in the map by comparing theoretical and experimental masses covered 95% of the sequence
- The peak patterns in the chromatograms are comparable among all the tested samples
- In addition a co-mix study was performed (data not shown)
- Heterogeneity due to posttranslational modifications at the heavy chain (Oligosaccharide pattern and C-terminal lysine removal) was confirmed
- No new peaks were detected in the new material, indicating that all samples had identical primary structure

Binding to Fcγ-receptors using Biacore

- To assess affinity of the antibody to a soluble form of recombinant human IgG Fcγ receptors the equilibrium binding was recorded.
- A surface plasmon resonance-based binding assay was established
- Equilibrium binding between the antibody and the recombinant Fc receptors immobilized on a CM5 sensorchip was recorded

Binding to Fcγ-receptors using Biacore

Binding *vs.* antibody concentration



FcγRIa (*high affinity*) → same affinity
FcγRIIa (*low affinity*) → same affinity
FcγRIIIb (ADCC) → new material has lower affinity

Antibody from new cell line has lower content of oligosaccharides with terminal galactose

Old process

New process

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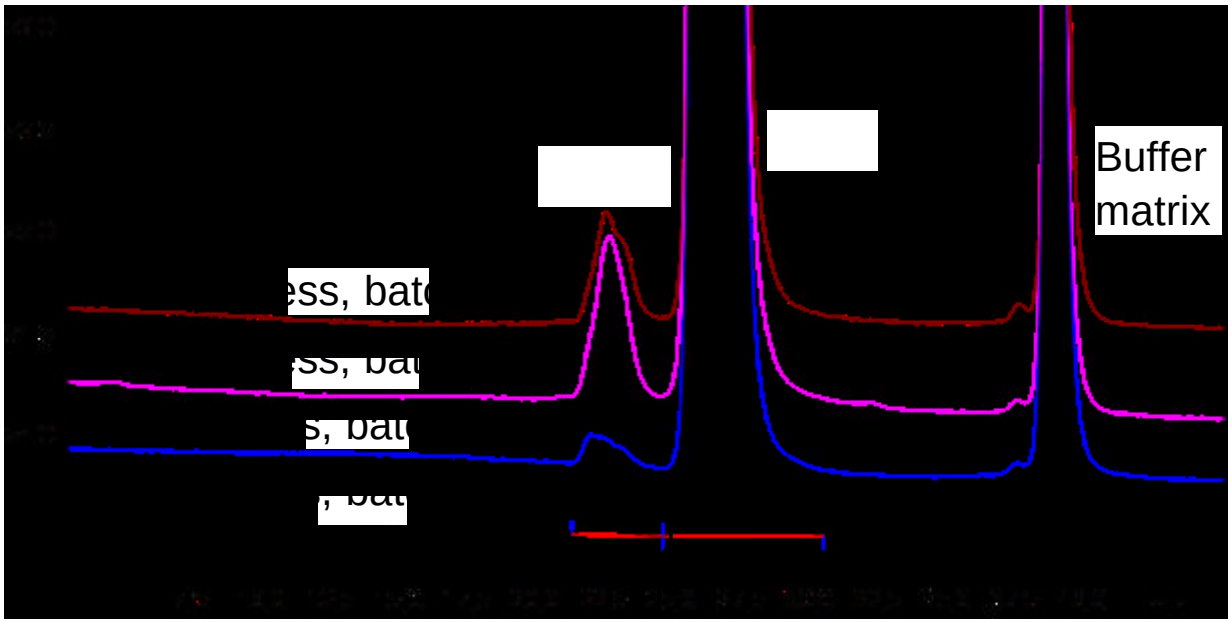
Impurities by Size Exclusion Chromatography

Impurities like aggregates or fragments were quantified



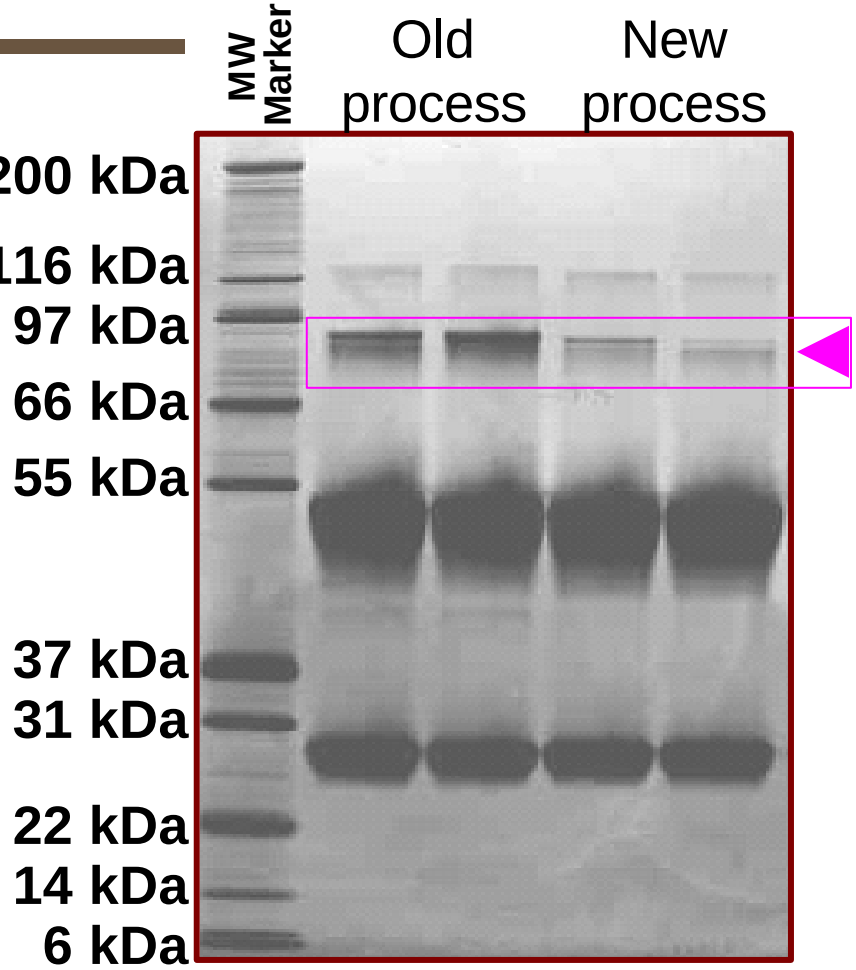
0.1% dimers

- No new component was detected in the new material.
- The amount of dimers was slightly increased to 0.4%, but still below the acceptance criterion (0.5%)

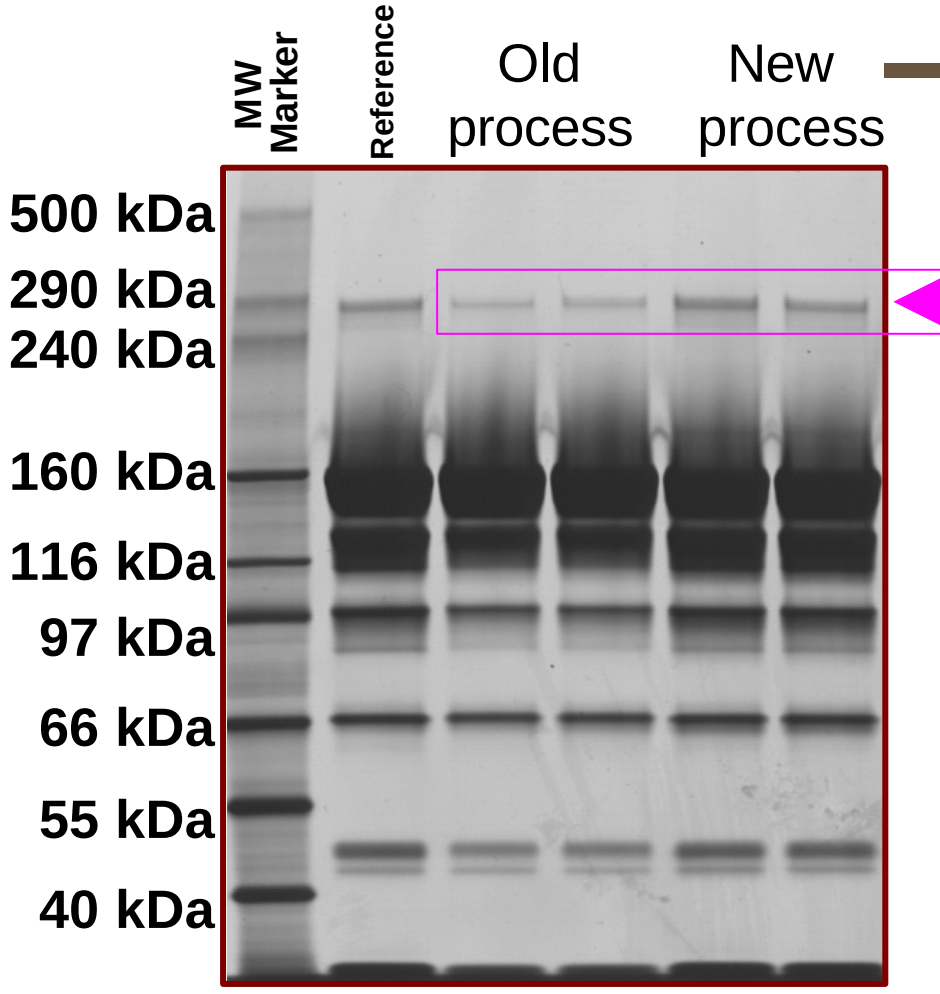
Impurities by SDS-PAGE (Silver Staining)

- SDS-PAGE allows separation of denatured proteins in an electric field based on their size
- The four antibody batches were analyzed under reduced and non-reduced conditions
- After electrophoresis, the gels were silver stained
- Proteins markers with known molecular weights were run in parallel

Impurities by SDS-PAGE (Silver Staining)



Reduced conditions



Non-reduced conditions

All batches exhibit same band patterns and similar purity levels, under both reduced and non-reduced conditions.

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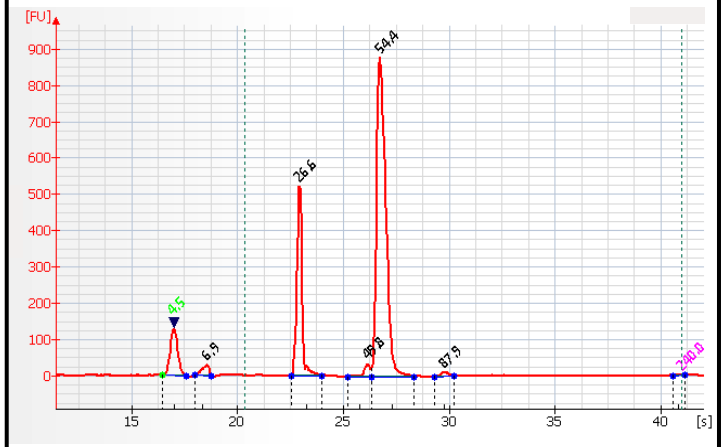
Impurities by Electrophoresis on the Chip (using the Bioanalyzer)

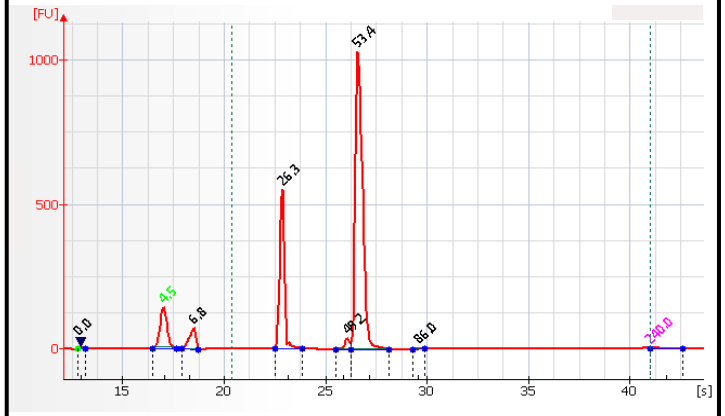
- The gel electrophoresis on the chip separates denatured proteins in an electric field on their size
- Impurities were detected by laser induced fluorescence after chip-based electrophoresis using an Agilent Bioanalyzer
- The antibody batches were analyzed under reduced and non-reduced conditions
- The molecular weight of proteins can be estimated by comparing to two internal standards in each injection

Impurities by Electrophoresis on the Chip (using the Bioanalyzer)

Old process:

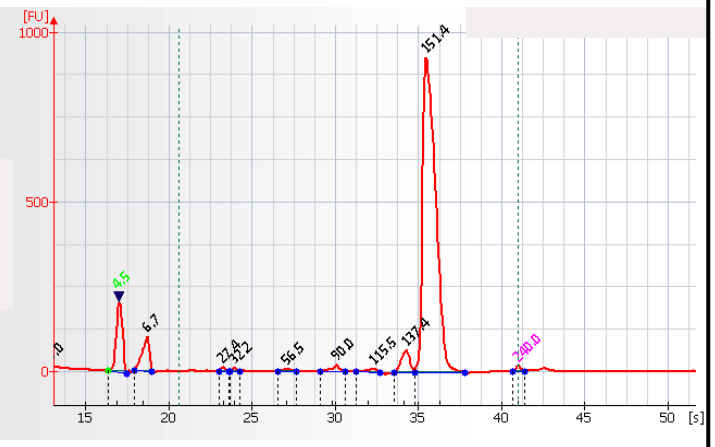


New process:

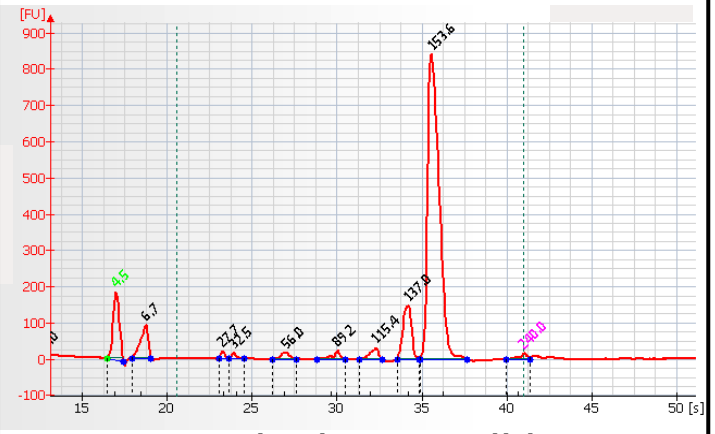


Reducing conditions

Old process:



New process:



Non-reducing conditions

No new impurities detected, profile is comparable

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Accelerated Stabilty Study

- Samples: -60°C, 25°C, and 40°C
  6 weeks, 3 months, 6 months
- Tests performed
  - Impurities by SEC, SDS-PAGE, IEF and CEX
  - Bioassay
  - pH, Color, and Turbidity
- Slightly increased level of dimeric aggregates in new material
- For old and new material the results from stability indicating tests are comparable

Overview of tests applied

Analytical technique	Result
Molecular mass of reduced antibody by LC-MS	Δ mass: 0.1Da (LC), 0.6Da (HC)
Mass of intact antibody by Q-TOF MS	Mass difference: 3 – 6 Da
N-terminal sequencing analysis	complies
Lys-C and Asp-N peptide mapping analysis	No new components found
Northern blot analysis and cDNA sequencing of total RNAs from the end of production cell banks	Corresponding amino acid sequences comply
Circular Dichroism spectroscopy	CD spectra match
Cell based QC bioassay (reporter gene assay)	New batches: 105% and 105%
Cell based bioassay from research (using human primary cells)	Relative IC50-values: 0.85 and 1.07
Binding assay to antigen using Biacore	Relative K_{on} –values: 1.04 and 0.95
Binding to soluble Fc-receptors using Biacore	Fc?RIa and Fc?RIIa: same affinity Fc?RIIIb: lower affinity

Overview of tests applied

Analytical technique	Result
Identity of glycan residues by MS	No new glycans found
Glycosylation profile	Different ratio of the main glycans
Size-Exclusion Chromatography (SEC)	Complies (dimer from 0.1% to 0.4%)
SDS-PAGE (reduced/non-reduced), silver staining	complies
Purity by Bioanalyzer (reduced / non-reduced)	complies
Reversed phase chromatography of reduced antibody	Similar patterns and levels of by- and degradation products found
Cation exchange chromatography	Same retention times for the main components No additional components detected
Isoelectric focusing	Complies

Summary

- To assess the impact of process changes including cell line switch, antibody batches from old and new process were characterized using physico-chemical and biological assays
- The capability of removing process-related impurities in the new process was comparable to the old process
- Oligosaccharide pattern differs as expected; does not affect pharmacokinetic behavior as shown in a separate study in monkeys (not shown).
- Slightly increased level of dimeric aggregates in new material
- Lower affinity of new material to Fc?RIIIb, that is not considered to be relevant for the biological activity, as an ADCC response is not required for the mode of action
- Stability data under stressed conditions are comparable
- Process changes have no impact on the product quality

Acknowledgements

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