Better biotherapeutic characterization for improved quality control

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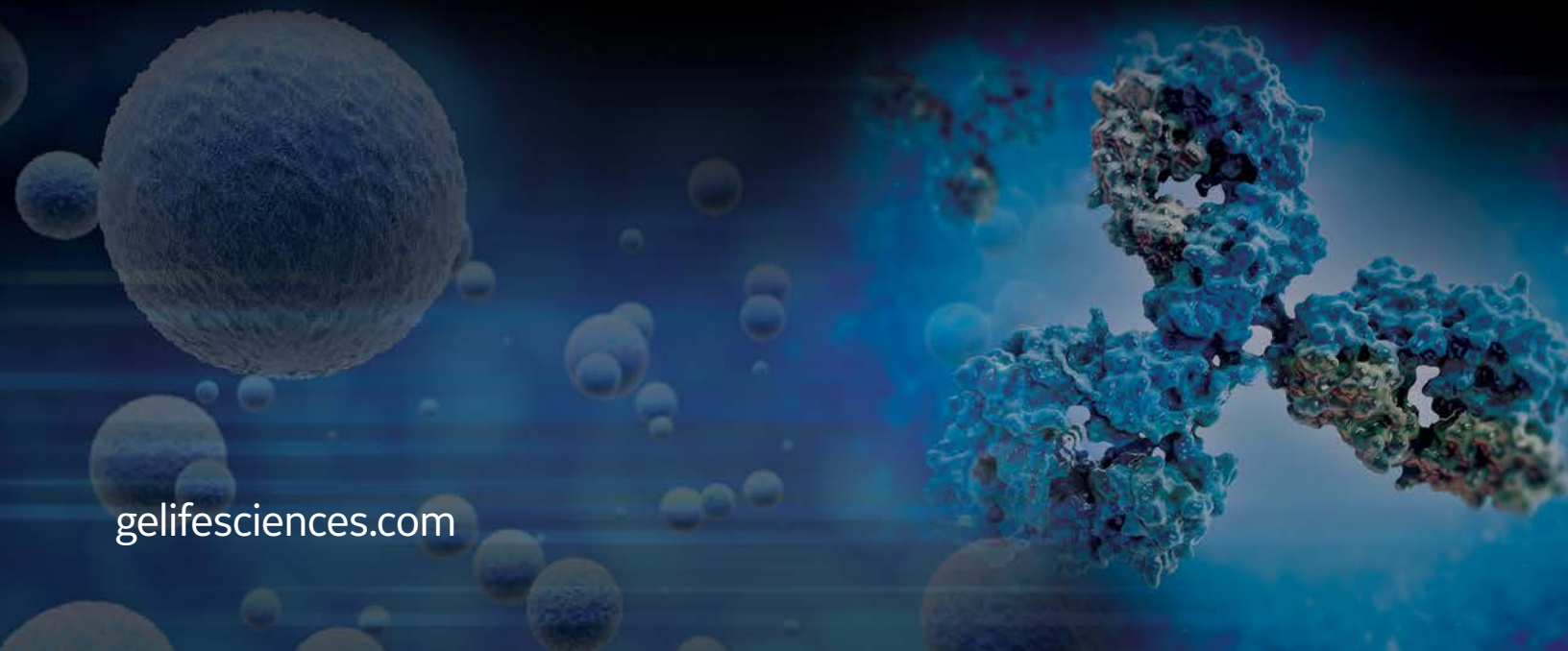
Better biotherapeutic characterization for improved quality control

Critical quality attributes (CQA) are fundamental to regulatory compliance and typically include data on how the candidate interacts with target proteins, size, and purity. In addition, information on process related CQA such as protein integrity, homogeneity, presence of host cell proteins, and DNA is required. This poster booklet provides a snapshot on how different analytical technologies have been used to look into aspects related to the efficacy, stability, and safety of therapeutic candidates.

Poster highlights

- Quantitate aggregates and/or protein fragments
- Surrogate potency assays with SPR—two CQA determined in only one assay
- Facilitating IgG FcRn analyses
- Reliable HCP detection



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Improved performance using Superdex™ 200 Increase 10/300 GL in product lot release

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Introduction

Superdex 200 10/300 GL size exclusion chromatography (SEC) columns are used as part of product lot release for several protein-based drugs, to quantify aggregates and/or protein fragments. Increases in both complexity of protein drugs and stringency of drug safety requirements are driving the demand for SEC columns with improved abilities to detect impurities. To raise the standards and meet these new needs, GE Healthcare has introduced a new generation of agarose-based SEC resins and columns with improved performance. Superdex 200 Increase resin has rigid small beads with narrow particle size distribution to provide high resolution in a short time. The narrower specifications for particle size distribution and selectivity compared with Superdex 200 result in higher lot-to-lot consistency and thus improved reproducibility.

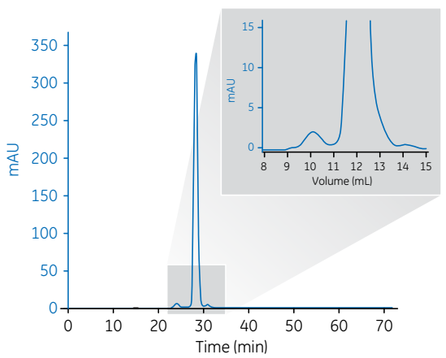
Improved resolution in mAb analyses

The combination of smaller beads and narrower particle size distribution of the Superdex 200 Increase resin compared to Superdex 200 resin allows higher resolution between molecules that are close in size. For example, Superdex 200 Increase improves the separation of monoclonal antibody fragments so that more fragments can be detected.

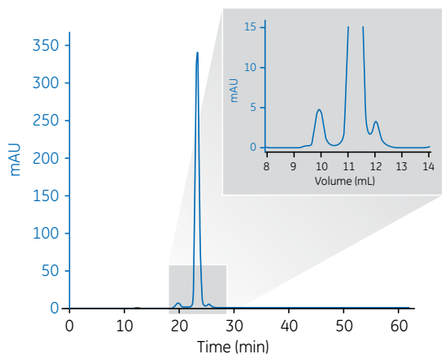
Running conditions

Columns:	Superdex 200 10/300 GL and Superdex 200 Increase 10/300 GL
Sample:	100 µL monoclonal antibody mAb5 (3.4 mg/mL)
Flow rate:	0.5 mL/min
Buffer:	20 mM NaH_2PO_4 pH 7.4, 300 mM NaCl
Detection:	UV 280 nm

Superdex 200 10/300 GL



Superdex 200 Increase 10/300 GL



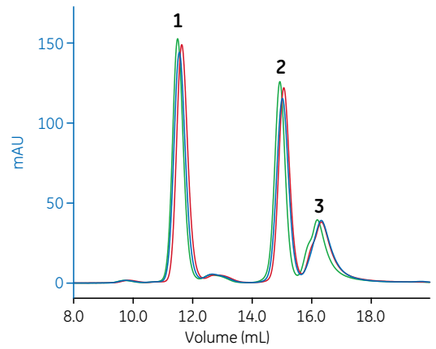
Column-to-column reproducibility

Three Superdex 200 Increase 10/300 GL columns packed with the same chromatography resin lot were compared by running a sample of mAb that contained both Fab and dAb.

The results show a high reproducibility in column packing, which ensures high consistency.

Running conditions

Columns:	Superdex 200 Increase 10/300 GL
Sample:	1. mAb5 (monomer) – M_r 150 000 2. Fab (mAb5) – M_r 50 000 3. dAb (mAb5) - M_r 13 000
Sample volume:	50 µL (0.2% CV)
Buffer:	20 mM NaH_2PO_4 pH 7.4, 300 mM NaCl
Flow rate:	0.75 mL/min
Detection:	UV 280 nm



Lot-to-lot reproducibility

Six Superdex 200 Increase 10/300 GL columns packed with six different lots of chromatography resins were tested. Differences in resolution and retention volume were measured for columns run in triplicate with a sample containing mAb, Fab, and dAb.

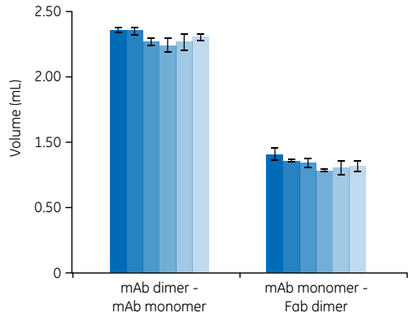
Results:

- Resolution – Minor differences, RSD[1] < 6 %
- Retention volume (mL) – Minor differences, RSD < 10 %

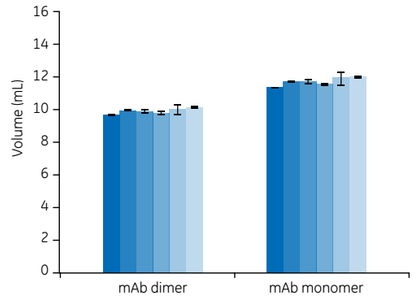
Running conditions

Columns:	Superdex 200 Increase 10/300 GL
Sample:	mAb5 (monomer) – M_r 150 000 Fab (mAb5) – M_r 50 000 dAb (mAb5) - M_r 13 000
Sample volume:	50 µL (0.2% CV)
Buffer:	20 mM NaH_2PO_4 pH 7.4, 300 mM NaCl
Flow rate:	0.75 mL/min
Detection:	UV 280 nm

Resolution



Retention volume



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Conclusions

Comprising small, rigid beads with a narrower particle size distribution and a higher selectivity than its predecessor, Superdex 200 Increase offers:

- Improved performance for mAb analysis
- High lot-to-lot consistency in resolution and retention volumes

Protein analysis and characterization with new generation agarose-based SEC columns

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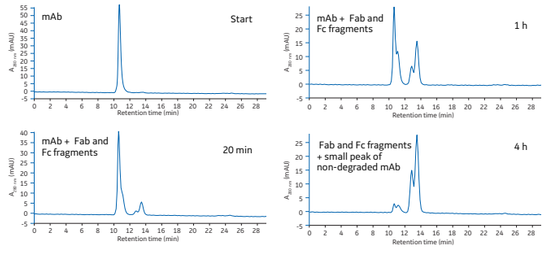
Introduction

Size-exclusion chromatography (SEC) is the standard method for analysis and characterization of protein aggregation or truncation. Such size changes can be studied under a number of conditions, for example, for storage analysis of biopharmaceuticals. With the introduction of the resin Superdex™ 75 Increase, agarose-based SEC columns are now available to cover the separation range of proteins from M_r 3000 to 5 000 000. The columns are designed for rapid separation and analysis of small proteins up to large protein complexes and other biomolecules.

High resolution detection of products after enzymatic cleavage with Superdex 75 Increase 10/300

Monitoring of mAb degradation into Fab and Fc fragments after papain cleavage.

Column: Superdex 75 Increase 10/300 GL
Samples: Products after different times of enzymatic cleavage (by papain) of mAb
Sample volume: 50 µL
Flow rate: 0.75 mL/min
Buffer: 10 mM sodium phosphate, 140 mM sodium chloride, pH 7.4
System: ÄKTA™ chromatography system
Time from start of the enzymatic reaction: At start; after 20 min, 1 h, and 4 h

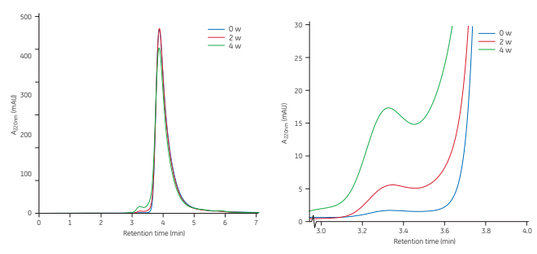


Resolution pattern after papain cleavage of mAb. During the enzymatic cleavage reaction the mAb is degraded into Fab and Fc fragments, as seen in the chromatograms above.

Efficient analysis of protein storage stability with Superdex 75 Increase 5/150 GL

Superdex 75 Increase 5/150 GL was used to rapidly monitor small changes in size homogeneity (formation of aggregations) in a large number of samples stored under different conditions and over different time periods (weeks).

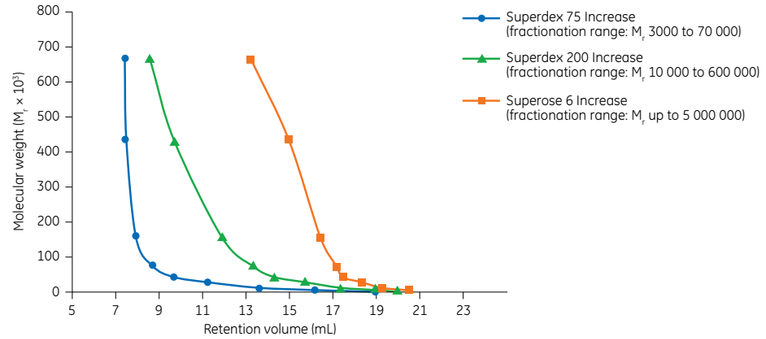
Column: Superdex 75 Increase 5/150 GL
Samples: Stored protein preparations
Sample volume: 10 µL
Flow rate: 0.5 mL/min
Buffer: 10 mM sodium phosphate, 140 mM sodium chloride, pH 7.4
System: HPLC system equipped with autosampler



Monitoring of size-homogeneity changes of proteins in the form of higher protein M_r variants during storage (0, 2, and 4 weeks) using Superdex 75 Increase 5/150 GL.

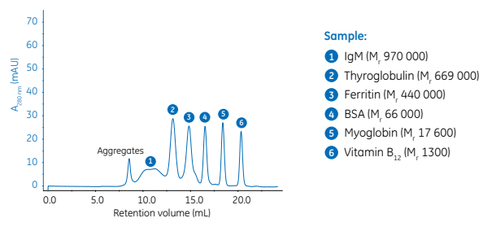
For other biomolecules, Superdex 200 Increase and Superose™ 6 Increase complement Superdex 75 Increase

Selectivity curves for Superose 6 Increase, Superdex 200 Increase, and Superdex 75 Increase, showing the broad fractionation range.



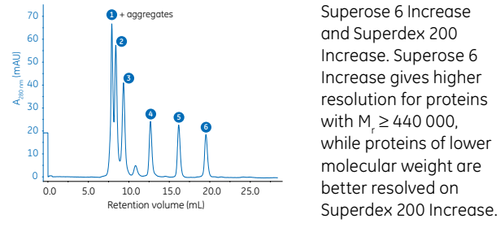
Elution volumes for globular proteins of various molecular weights for the different resins packed in 10/300 columns
Note: The whole fractionation range of Superose 6 Increase is not covered in this diagram.

Superose 6 Increase 10/300 GL



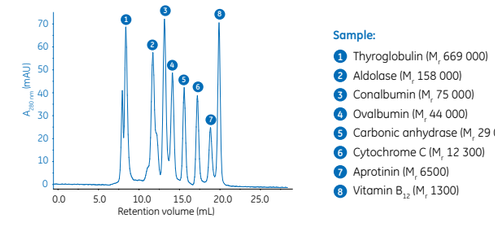
Sample:
1. IgM (M_r 970 000)
2. Thyroglobulin (M_r 669 000)
3. Ferritin (M_r 440 000)
4. BSA (M_r 66 000)
5. Myoglobin (M_r 17 600)
6. Vitamin B_{12} (M_r 1300)

Superdex 200 Increase 10/300 GL



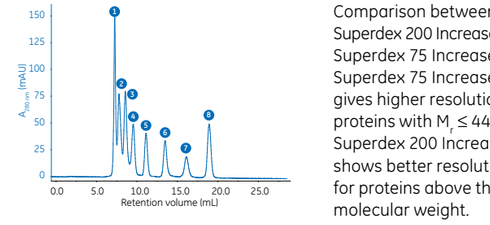
Comparison between Superose 6 Increase and Superdex 200 Increase. Superose 6 Increase gives higher resolution for proteins with $M_r \geq 440\,000$, while proteins of lower molecular weight are better resolved on Superdex 200 Increase.

Superdex 200 Increase 10/300 GL



Sample:
1. Thyroglobulin (M_r 669 000)
2. Aldolase (M_r 158 000)
3. Conalbumin (M_r 75 000)
4. Ovalbumin (M_r 44 000)
5. Carbonic anhydrase (M_r 29 000)
6. Cytochrome C (M_r 12 300)
7. Aprotinin (M_r 6500)
8. Vitamin B_{12} (M_r 1300)

Superdex 75 Increase 10/300 GL



Comparison between Superdex 200 Increase and Superdex 75 Increase. Superdex 75 Increase gives higher resolution of proteins with $M_r \leq 44\,000$. Superdex 200 Increase shows better resolution for proteins above this molecular weight.

Conclusions

Superdex 75 Increase allows:

- analysis of biomolecules in the range of M_r 3000 to 70 000
- generation of high resolution data
- rapid and efficient analysis with the 5/150 GL column format

For biomolecules of other sizes, Superdex 200 Increase and Superose 6 Increase columns are also available.

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Preparation and high resolution analysis of a Fab

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Introduction

Antibody fragments are getting increased attention in research use due to their small size and lower immunogenicity (relative to intact antibodies).

In this work Fab was produced by papain cleavage of a purified monoclonal antibody (mAb) followed by purification on MabSelect Sure™ and Capto™ L resins (Protein A and Protein L immobilized to high flow agarose, respectively). Fractions from the different steps were analyzed with a new generation size exclusion chromatography (SEC) resin, Superdex™ 200 Increase.

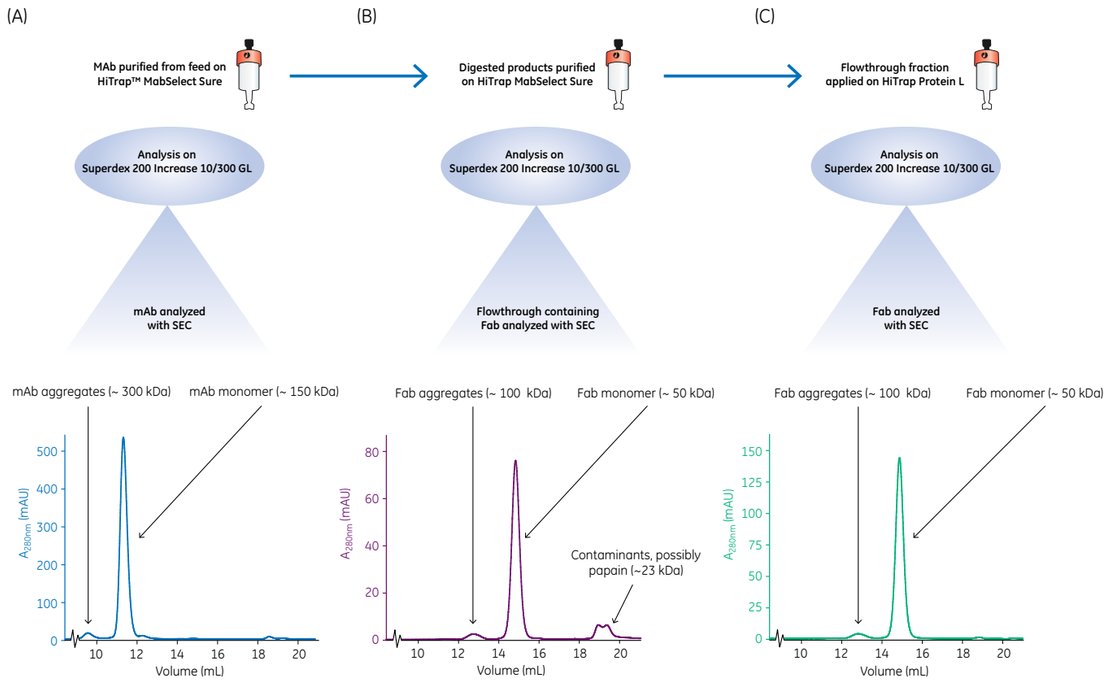

Fig 1. SEC analyses of (A) Purified mAb (B) Flowthrough containing Fab, and (C) Purified Fab. Affinity chromatography run on HiTrap 5 mL MabSelect Sure and Protein L. Buffers and flow rates according to resins instructions. All SEC analyses were run on Superdex 200 Increase 10/300 GL with PBS, 0.5 mL/min and 50 µL sample on ÄKTA™ pure chromatography systems.

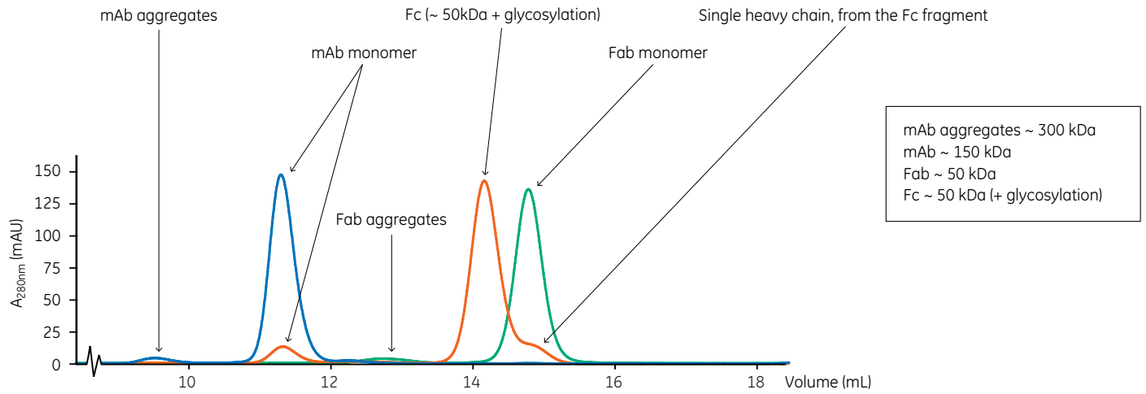

Fig 2. Overlay of individual SEC analyses of mAb, Fab, and Fc fragments. For comparative purposes, the curves have been normalized against the peak height of each monomer species.

Conclusions

- HiTrap MabSelect Sure and HiTrap Protein L enable a simplified workflow to produce Fab by papain digestion of mAb
- High resolution power of Superdex 200 Increase allows baseline separation between monomer and aggregates from both mAb and Fab
- Broad separation range (M_r 10 000 to M_r 600 000) with optimized resolution for antibodies (M_r 100 000 to M_r 300 000) allows separation of a large number of different biomolecules

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Surrogate potency assays with SPR—two CQA determined in only one assay

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Introduction

An array of potency assays is needed to measure and monitor critical quality attributes (CQA) of antibody therapeutics. We present how Biacore™ surface plasmon resonance (SPR)-based assays can be used as surrogate potency assays to facilitate comparability and biosimilar studies (1). A capture-based assay that can be expanded to include estimates of relative potency for several CQAs in a single experiment is described. We also demonstrate how *Sensorgram comparison* complements dose response curves to ensure compliance with kinetic properties.

Surrogate potency assay

Setup and robustness: exemplified for anti-TNF-α antibodies

Biacore Biotin CAPture Kit was used rather than covalent coupling, minimizing assay development time.

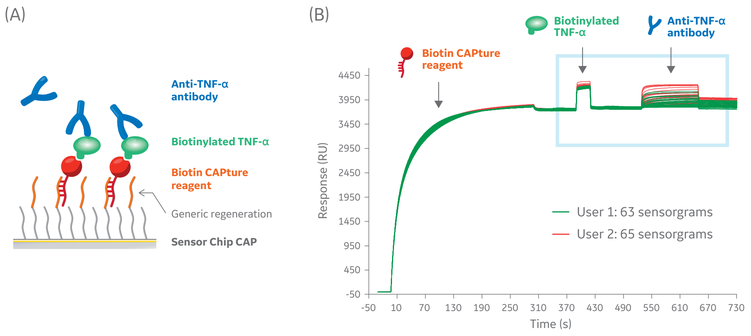


Fig 1. (A) The sensor chip surface was functionalized by injection of a streptavidin oligo conjugate Biotinylated TNF-α was then captured to the surface followed by antibody binding. (B) Overlay plot showing more than 120 cycles from two different users, illustrating the robustness of the assay. After each injection the surface was regenerated in preparation for a new injection.

Construction of potency curve

Obtaining reproducible capture levels is crucial for potency analysis and here 0.5% BSA was added to the TNF-α buffer. PBS-P+ without BSA was used as running and antibody buffer.

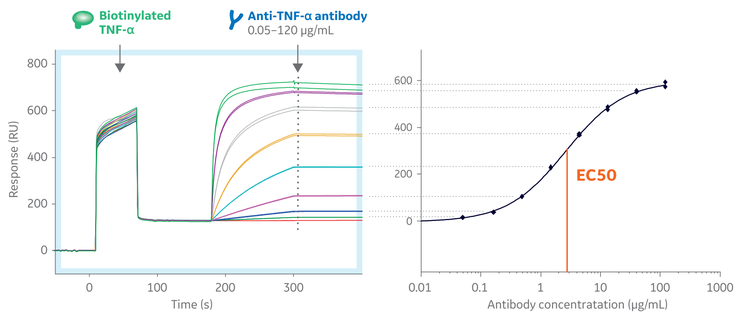
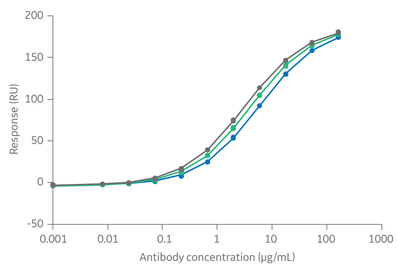


Fig 2. Construction of a potency curve. Responses taken after end of antibody injections (dashed vertical line) were plotted against concentration and a four-parameter fitting of data was used.

The assay is fit for purpose



Sample relative conc. (%)	EC50 (µg/mL)	Relative potency (%)
100 (reference)	4.23	100.0
130	3.30	128.1
70	5.90	71.7

Fig 3. Varying nominal concentrations shows that EC50 values are linear with respect to concentration.

References

1. Karlsson R. *et al.* Kinetic and specific concentration information strengthens confidence in relative potency analyses of antibody critical quality attributes. *J. Pharm. Anal.* Submitted for publication (2017).
2. Karlsson R. *et al.* Comparison of surface plasmon resonance binding curves for characterization of protein interactions and analysis of screening data. *Anal. Biochem.* **502**, 53–63 (2016).

Results

Potency data for two CQA obtained in one experiment

Antibody and Fcγ receptor bind in sequence resulting in two potency curves from the same experiment.

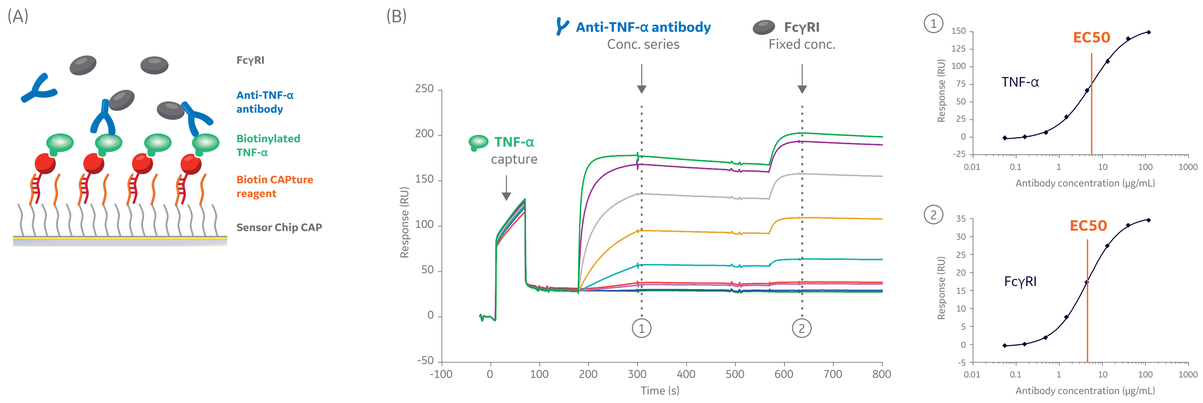


Fig 4. (A) Anti-TNF-α antibody and FcγRI bind in sequence. (B) Responses (dashed lines) were plotted against antibody concentration. Potency curves for antibody binding to both TNF-α and FcγRI were obtained in the same experiment.

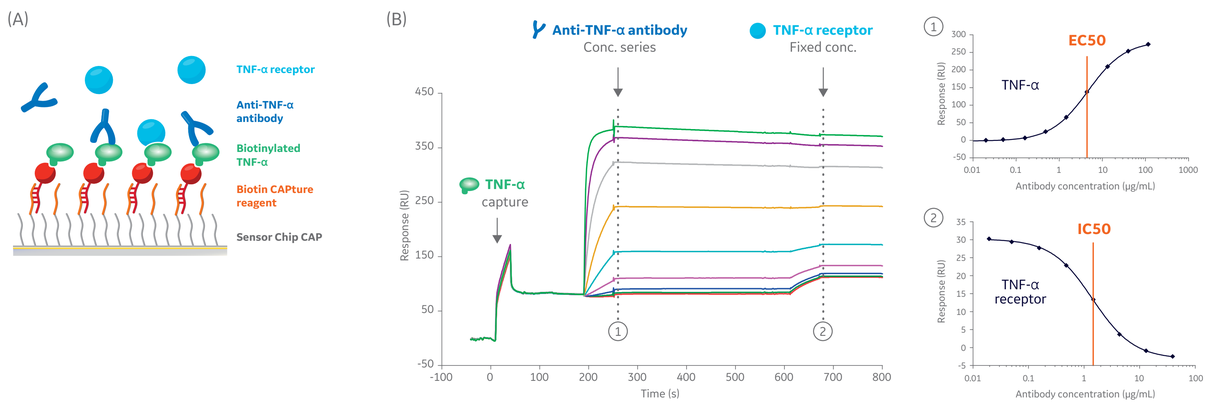


Fig 5. (A) Anti-TNF-α antibody and TNF-α receptor compete for binding to captured TNF-α. (B) Responses (dashed lines) were plotted against antibody concentration. Two potency curves were obtained from the same experiment as was the case in Figure 4. Here, however, TNF-α receptor binding resulted in an inhibition curve and an IC50 value (curve 2).

Dose-response curves from Biacore assay, ELISA, and other techniques do not provide the whole story

Curve shifts depend not only on concentration but also on kinetic parameters

Simulated data demonstrate how kinetic properties can shift the potency curve. Data mimics the stable binding between anti-TNF-α antibody and TNF-α with rate constants: $k_a = 1.6 \times 10^6\ M^{-1}\ s^{-1}$; $k_d = 8.5 \times 10^{-5}\ s^{-1}$.

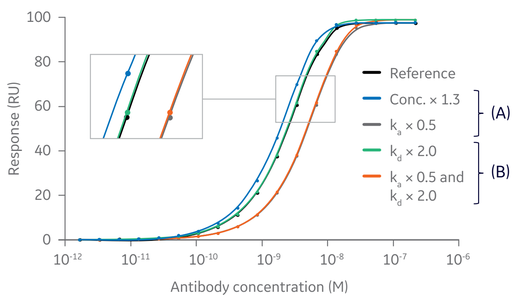


Fig 6. Potency curve shifts depend on concentration and kinetic parameters. (A) Differences in concentration and in association rate shift the curve and EC50. (B) Changing the dissociation rate does not shift the curve.

↓

Differences in dissociation rates can be missed for stable binders

Sensorgram comparison complements potency data

By prolonging the dissociation time for a selected concentration during potency analyses, differences in dissociation can be detected. *Sensorgram comparison* (2) describes this: a comparison window is defined by the user, allowing for some variation of the reference. Samples falling within the window are accepted (Similarity score = 100%), while samples falling outside need further analysis, and may be ranked according to decreasing similarity percent.

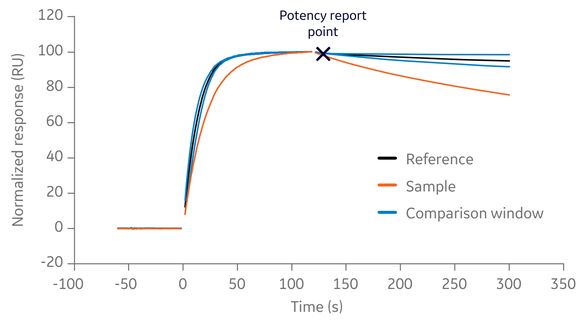


Fig 7. Potency assay complemented with *Sensorgram comparison*. The sample (red curve) obtains similar potency response (✕) as the reference but appears *outside* the comparison window when dissociation is prolonged. Kinetic profiles for reference and sample differ.

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Differences in kinetic profiles can be detected

Conclusions

- A Biacore SPR-based surrogate potency assay is described. The assay uses a capture format, which minimizes time for assay development.
- The assay can be extended to obtain potency data for at least two CQA in the same experiment, and is applicable also to, for example, bispecifics.
- Dose-response curves for Biacore assay, ELISA, and other techniques give limited information on similarity, especially for strong binders. By including the Biacore software functionality *Sensorgram comparison* in potency data evaluation, differences in kinetics can be detected.

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Facilitating IgG FcRn analyses

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Introduction

Therapeutic antibodies are approved for a number of different indications. One important antibody attribute is the Fc Receptor (FcRn) binding properties.

In the present study an assay setup combining biotin capture of FcRn with single-cycle kinetic analysis of the antibody interaction is described.

Further the use of *Dual/ABA inject* for easy analyses of pH dependency is introduced.

The binding mechanism between antibodies and FcRn is heterogeneous due to avidity rendering kinetic analysis of the interaction complex. A simplified statistical approach to SPR analysis, without assuming a particular binding mechanism, is here used for antibody/FcRn analyses.

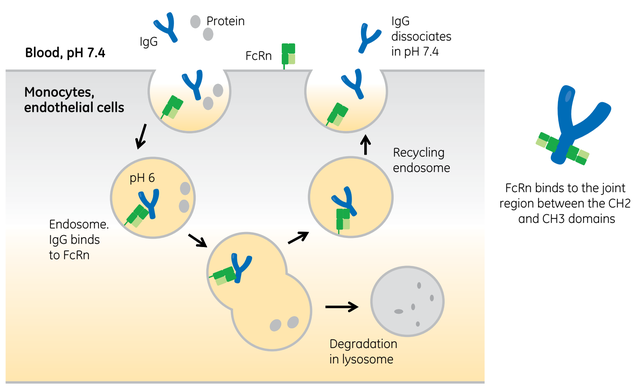


Fig 1. FcRn protects IgG from lysosomal degradation. After pinocytosis IgG binds to FcRn in the endosome and is recirculated out into the blood, thus prolonging the half-life of IgG. Two FcRn molecules are assumed to bind to each IgG Fc with similar affinity (1).

Assay set up using Biotin CAPture Kit

Biotin CAPture Kit:

- Stable, oriented capture of biotinylated FcRn
- Easy to control capture level and change type of FcRn
- Capture kit adds convenience, minimal assay development

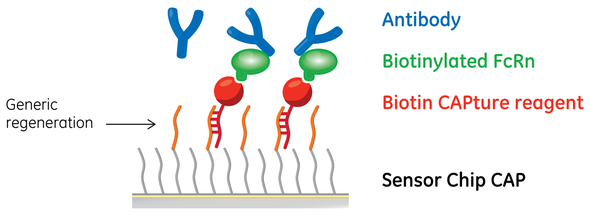


Fig 2. Schematic view of the chip surface of Sensor Chip CAP with immobilized deoxyribo-oligonucleotides. Biotin CAPture Reagent (Streptavidin) is first hybridized to the deoxyribo-oligonucleotides, biotinylated FcRn is captured and analysis of antibody binding performed. The arrow indicates where the regeneration will act on the chip surface.

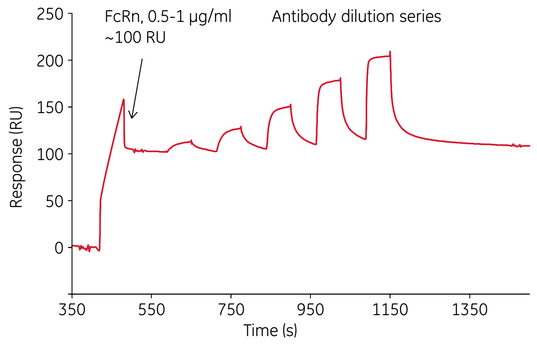


Fig 3. Capture of approx. 100 RU biotinylated FcRn followed by injection of antibody concentration series.

Detailed assay conditions

- CAP reagent, ready-to-use, 5 min injection over both flow cells, obtaining approx. 3000 RU
- Biotinylated FcRn (Immunitrack, Denmark) 0.5-1 µg/ml injected 60 s over flowcell (Fc) 2, to obtain approx. 80-100 RU
- Antibody, single-cycle kinetics 60 s with 300 s dissociation time, over Fc 1 and 2
- Assay buffer: 20 mM phosphate, 150 mM NaCl, 0.05% Surfactant P20, pH 6.0 or 7.4, depending on purpose
- Regeneration: 120 s regeneration solution, Fc 1 and 2. (mix of guanidine hydrochloride and NaOH)

References

1. Abdiche, Y. N. *et al.* 2014, mAbs, 7, 331-338.
2. Neuber, T. *et al.* 2014, mAbs, 6, 928-942.
3. Ober, R.J. *et al.* 2001, Int. Immunol., 13, 1551-9.

Antibody binding profiles for different FcRn species, Biacore T200

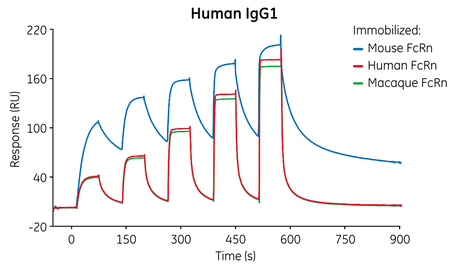


Fig 4. Human IgG1 (25-2000 nM) binding to approx. 100 RU captured FcRn from all species. Binding to mouse FcRn is significantly stronger than to human and macaque FcRn, binding to the latter two being very similar.

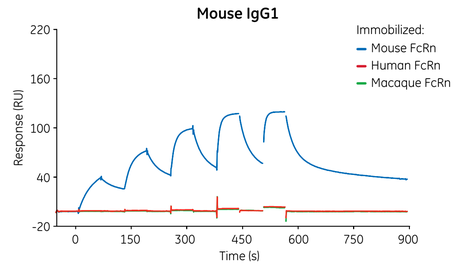


Fig 5. Mouse IgG1 (25-2000 nM) binding only to mouse FcRn (approx. 100 RU captured).

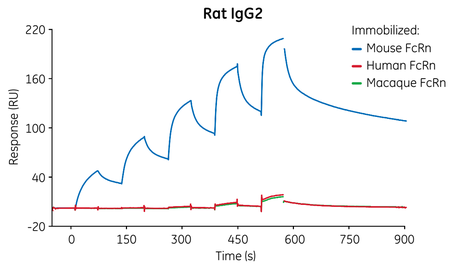


Fig 6. Rat IgG2 (25-2000) binding only to mouse FcRn (approx. 100 RU captured).

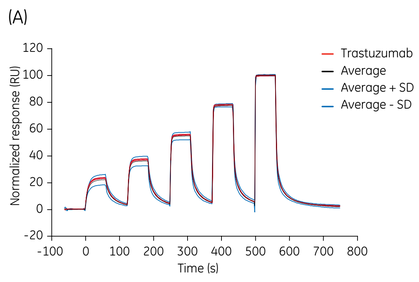
→ ***Binding specificities for all antibodies were in agreement with published data (1-3)***

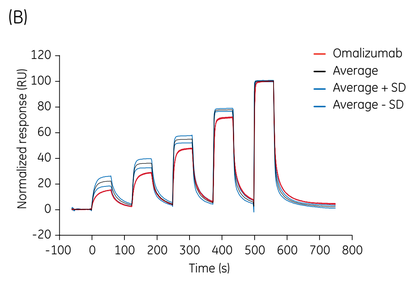
Quantitating similarities using the *Sensorgram Comparison* functionality, Biacore T200

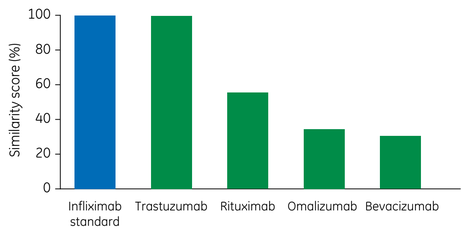
Sensorgram Comparison is an approach to data analysis where the whole, or part of the binding curve is used in the analysis. Sample data is compared to the performance of a standard, here infliximab. The expected variation of the standard data is statistically defined by a comparison window based on upper and lower limit sensorgrams.

A sample that falls inside the comparison window obtains a similarity score of 100%. The further away, i.e. the more deviating a sample is from the comparison window the lower the similarity score.

Results example; hFcRn binding profiles of therapeutic antibodies compared to that of infliximab.







Ligand	Sample	Similarity score (%)
hFcRn	Infliximab standard	99.92
hFcRn	Trastuzumab	99.72
hFcRn	Rituximab	55.45
hFcRn	Omalizumab	34.34
hFcRn	Bevacizumab	30.42

Fig 7. Infliximab was selected as standard (conc. 25–2000 nM), shown as black average curves in (A) and (B) surrounded by 3 SD wide comparison windows, blue. Both association and dissociation were compared in this example. The binding curve of trastuzumab (A, red) fell within the window and trastuzumab received a very high similarity score. In contrast omalizumab (B) has significantly slower on rate and slightly different off rate (red curve) here receiving a similarity score of 34.34%, using these settings.

pH dependent binding analyses facilitated using *Dual inject* or *ABA inject*

Principle using *Dual inject*, Biacore T200

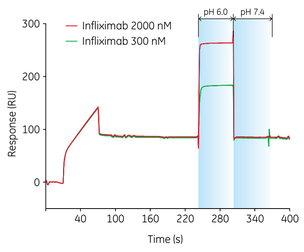


Fig 8. Capture of hFcRn and binding of infliximab (300 and 2000 nM in pH 6.0) followed by immediate dissociation in pH 7.4. Running buffer and FcRn capture buffer was PBS-P+, pH 7.4.

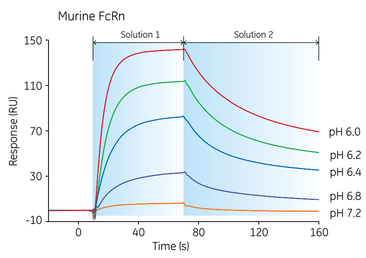


Fig 9. Infliximab (100 nM) interacting with hFcRn, each cycle having the same pH for binding and dissociation.

Principle using *ABA inject*, Biacore 8K

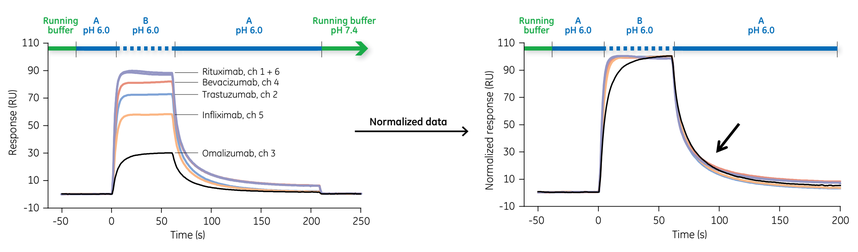


Fig 10. *ABA inject* used to inject 5 antibodies (100 nM in pH 6.0) binding to hFcRn with rituximab replicates in channel 1 and 6. All antibody injections were flanked by buffer, pH 6.0.

Fig 11. Data from Figure 10, normalized between 0 and 100. The antibodies show very similar dissociation at pH 6.0 with omalizumab differing slightly.

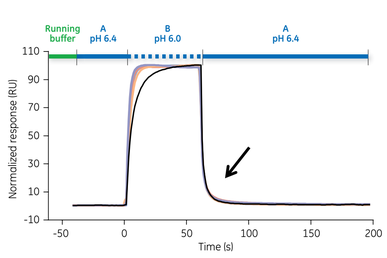


Fig 12. Binding in pH 6.0 with flanking buffer pH 6.4. Faster dissociation for all antibodies compared to data in Figure 11.

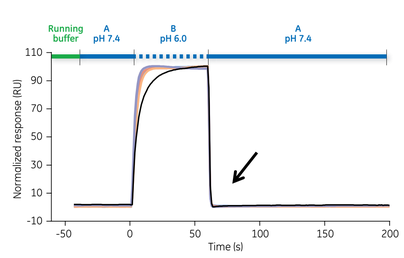


Fig 13. Binding in pH 6.0 with pH 7.4 as flanking buffers. Instant dissociation of all antibodies in buffer pH 7.4.

Biacore T100/T200	*Dual inject*
Biacore 2000/3000	*Co-inject*
Biacore S200/8K	*ABA inject*

- Running buffer remains the same, e.g. PBS-P+, pH 7.4
- *Dual inject and ABA inject* solutions are selected freely
- Many antibody analyses may be performed on the same captured FcRn
- Blanks with corresponding pH combinations were included at intervals
- Evaluation: association and/or dissociation rates visually compared

Biacore 8K
- **8 antibodies**
- **3 different pHs**
- **40 min run**

Conclusions

- Minimal assay development using Biotin CAPture Kit for capture of biotinylated FcRn. In combination with single-cycle kinetics both reagents and time are saved.
- *Sensorgram Comparison* quantifies the similarities between a standard and a sample without assuming a binding mechanism, an advantage for heterogeneous interactions like antibody/FcRn.
- *Dual inject* and *ABA inject* facilitates studies of pH dependent binding.
- With a large number of antibodies the time/antibody decreases significantly thanks to flexible use of channels in Biacore 8K.

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Biacore™ 8K+ for concentration analysis with throughput and precision

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GE Healthcare Bio-Sciences AB, Björkgatan 30, 751 84 Uppsala, Sweden

Introduction

The measurement of protein concentration in an aqueous sample is an important assay in biochemistry research and development labs for applications ranging from enzymatic studies to providing data for biopharmaceutical lot release.

Biacore 8K and Biacore 8K+ with its eight needles opens up for assays using a parallel calibration curve as well as the serial concentration analysis. The use of a parallel calibration curve provides shorter run times, which results in data with very good precision enhanced by interchannel normalization.

This poster presents the new software features in Biacore Insight Evaluation Software that supports concentration analysis with both serial and parallel calibration curves for data generated in Biacore 8K, Biacore 8K+ and Biacore T200. In Biacore 8K and Biacore 8K+, full GxP support is offered for runs and evaluations.

Biacore 8K+

Biacore 8K+ is a high-capacity surface plasmon resonance system, efficiently delivering binding data of outstanding quality, meeting your toughest challenges in screening, characterization, process optimization, and quality control. This eight needle, high-sensitivity SPR system rapidly provides kinetics, affinity, concentration, and potency data, shortening time to results by up to eight times compared to single-needle systems.

The extended sample hotel of Biacore 8K+ holds up to 12 microplates and the buffer selector allows for use of up to 4 different buffers.

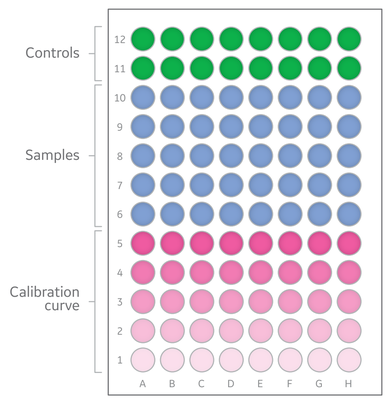
Biacore system and concentration analysis

Concentration analysis can be performed using calibration curves for each channel separately (serial approach) or with a calibration curve common for all channels (parallel approach). The parallel approach involves a channel normalization step to compensate for response differences between the channels, that is, all the responses from the calibration curve, control samples and unknown samples are adjusted.

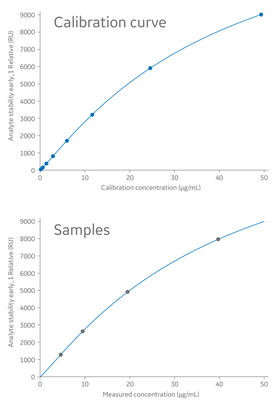
Serial concentration analysis

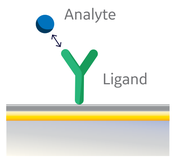
- Providing data with excellent precision
- Each channel holds its own calibration curve
- Beneficial when analyzing a large number of samples and/or high precision is required

Experimental setup



Results channel 1 (example)



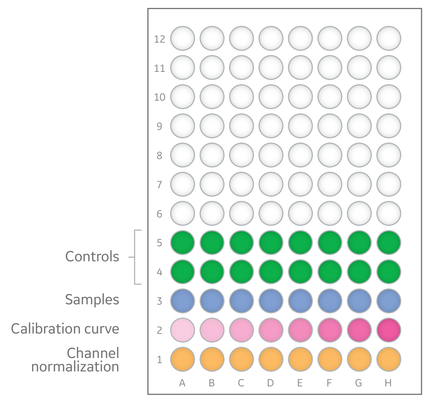


Direct binding assay

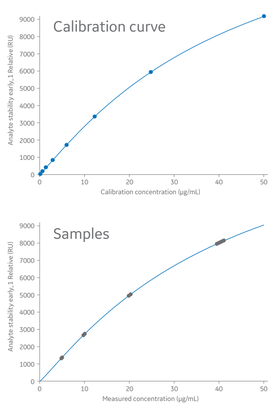
Parallel concentration analysis

- Providing data with very good precision
- Calibration curve across channels, only one cycle needed for calibration curve
- Shorter run time than serial concentration analysis
- Channel normalization accounts for differences in response from the used channels

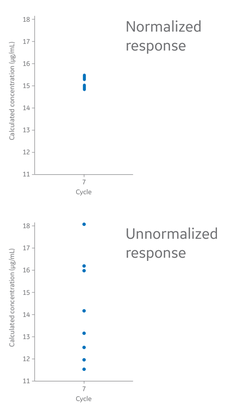
Experimental setup



Results channel 1-8 (example)



Channel normalization



The ligand used in the experiments was protein A (Sensor Chip Protein A, GE Healthcare) and the analyte was human IgG

Application example

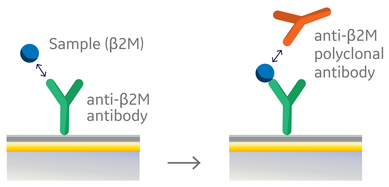
A concentration analysis to quantitate β2-microglobulin (β2M) in human plasma samples was performed using Biacore 8K+ set up with a parallel calibration curve. Comparison with an ELISA was made using identical sample and standard.

Experimental setup

Workflow overview 96-well plate with 40 samples (in duplicate)

Biacore assay

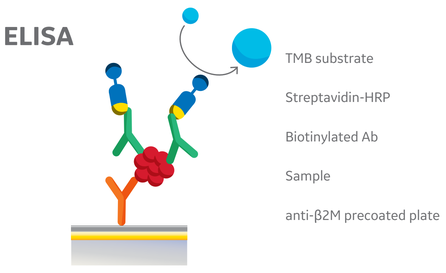
An enhancement molecule (anti-β2M polyclonal antibody) was included to increase the specificity of analyte binding due to issues with nonspecific binding from the plasma samples to the ligand (anti-β2M antibody). However, nonspecific binding to the dextran matrix was ruled out in a separate control experiment (not shown).



1	Dock a new sensor chip and immobilize
2	Prepare all reagents, samples and standard
3	Add all reagents, standard and sample to wells according to the plate layout in the predefined Biacore method. Cover and start the Biacore run.
4	Evaluate result

Assay preparation 2 h incl. surface preparation/coating.
Total assay time: 3.25 h
Total time to results: 5.25 h

ELISA



1	Prepare all reagents, samples and standard
2	Add standard and sample to wells. Cover and incubate at RT for **2.5 h**
3	Wash plate four times
4	Add biotinylated Ab to wells. Cover and incubate at RT for **1 h**
5	Wash plate four times
6	Add Streptavidin-horseradish peroxidase (HRP) reagent to each well. Cover and incubate at RT for **45 min**
7	Wash plate four times
8	Add tetramethylbenzidine (TMB) to each well
9	Develop plate at room temperature in dark for **30 min**
10	Add stop solution to each well
11	Measure absorbance and read results

Assay preparation 2 h excl. microplate coating.
Total assay time: Less than 5.5 h
Total time to results: 7.5 h (excl. microplate coating)

Benefits of Biacore analysis compared to ELISA

- Automated real-time determination of active concentrations with increased precision
- Saves on development time—no labeling of secondary reagents and enhancements might not be needed
- Easier sample preparation and ability to queue several methods and runs in sequence
- Reduced labor intensity—higher degree of automation and all hands-on activities performed in one step
- Accurate quantitation and/or affinity analysis of low-affinity/high K_D analytes often missed by ELISA

Conclusions

- Biacore 8K and Biacore 8K+ offer tools that allow for efficient concentration determination
- Biacore Insight Evaluation Software Concentration and Potency Extension package supports concentration analysis with both serial and parallel calibration curves
- The precision of concentration analysis using the parallel calibration curve is enhanced by channel normalization
- Concentration analysis assays performed in Biacore 8K, Biacore 8K+, and Biacore T200 can easily be evaluated in Biacore Insight Evaluation Software with the extension package
- The large hotel of Biacore 8K+ combined with the concentration analysis functionality enables a higher degree of automation compared to ELISA

KA4277060618PO

Enhanced HCP Coverage Analysis utilizing Multiplexed 2D Electrophoresis

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[1]Rockland Immunochemicals Inc., Limerick, PA 19646; [2]GE Healthcare Life Sciences, Uppsala, Sweden;
[3]Geneva Bioinformatics (GeneBio) SA, Geneva, Switzerland

Introduction

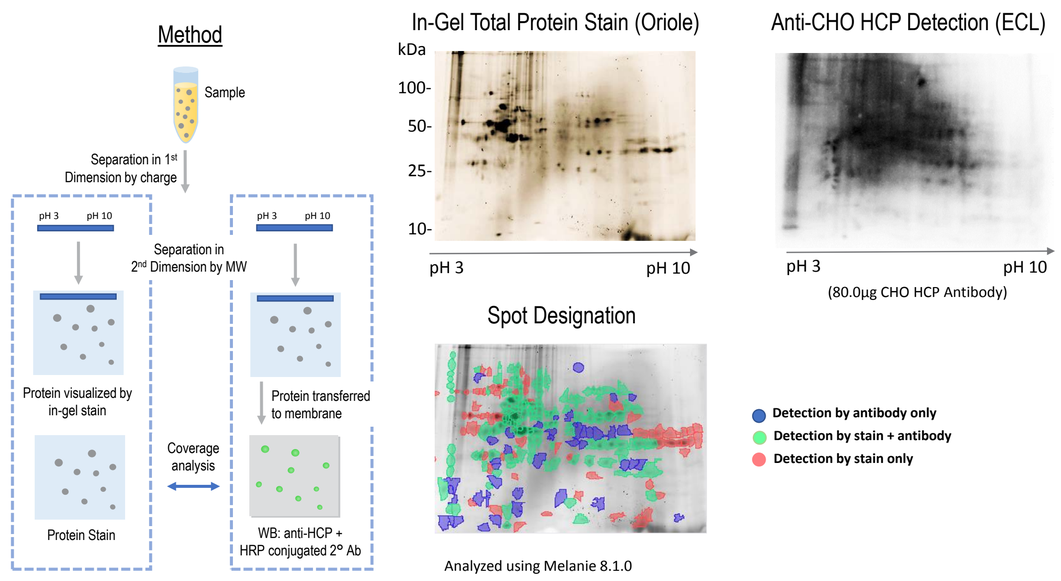
A crucial prerequisite in the drug manufacturing process is an efficient analysis of Host Cell Protein (HCP) impurities that result from process specific expression conditions as well as downstream purification procedures. Present guidelines call for minimum levels of HCP contaminants left behind during the purification process from the expression hosts. To investigate the presence of residual contamination of the final biopharmaceutical product, the development of polyclonal antibodies with maximum coverage against native HCP lysate provides a valuable tool to demonstrate product purity.

Quality of the HCP specific antibody is most commonly analyzed via ELISA and orthogonal methods like 1D and 2D-PAGE assays where the coverage of the antibody is confirmed. Determining anti-HCP antibody coverage is essential to ascertaining the robustness of the antibody in comprehensive reactivity toward HCPs during process points.

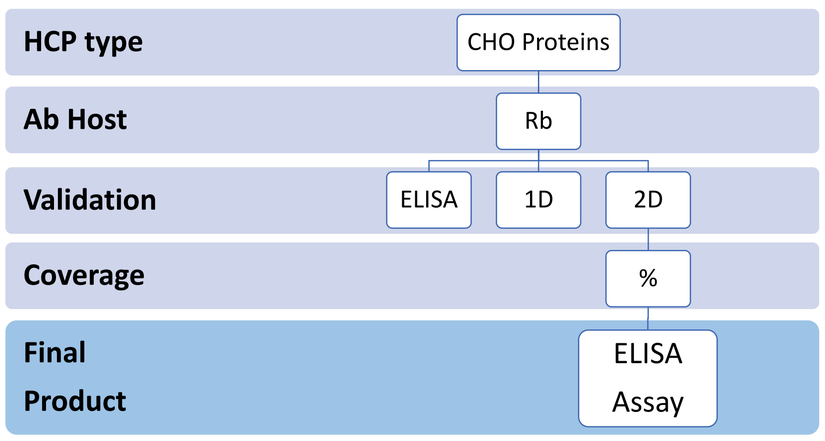
Here we evaluate the coverage of a generic anti-CHO-HCP antibody (Rockland Inc.) by 2D-PAGE separation using two experimental methods for the analysis: (A) chemiluminescent based detection method; and (B) Two-dimensional Differential In Blot Electrophoresis (2D-DIBE). For (A): the proteins detected by the antibody on a Western Blot (WB) via a chemiluminescent detection system, are equated to the proteins separated on a separate SDS-gel and visualized by an in-gel protein stain. For (B): 2D-DIBE utilizes a fluorescent multiplexing approach where the proteins and antibodies are tagged with different CyDyes™. Proteins and antibody coverage can be analyzed on the same membrane.

Sensitivity and antibody reactivity of the two methods are compared and analyzed.

2D-Chemiluminescence coverage detection



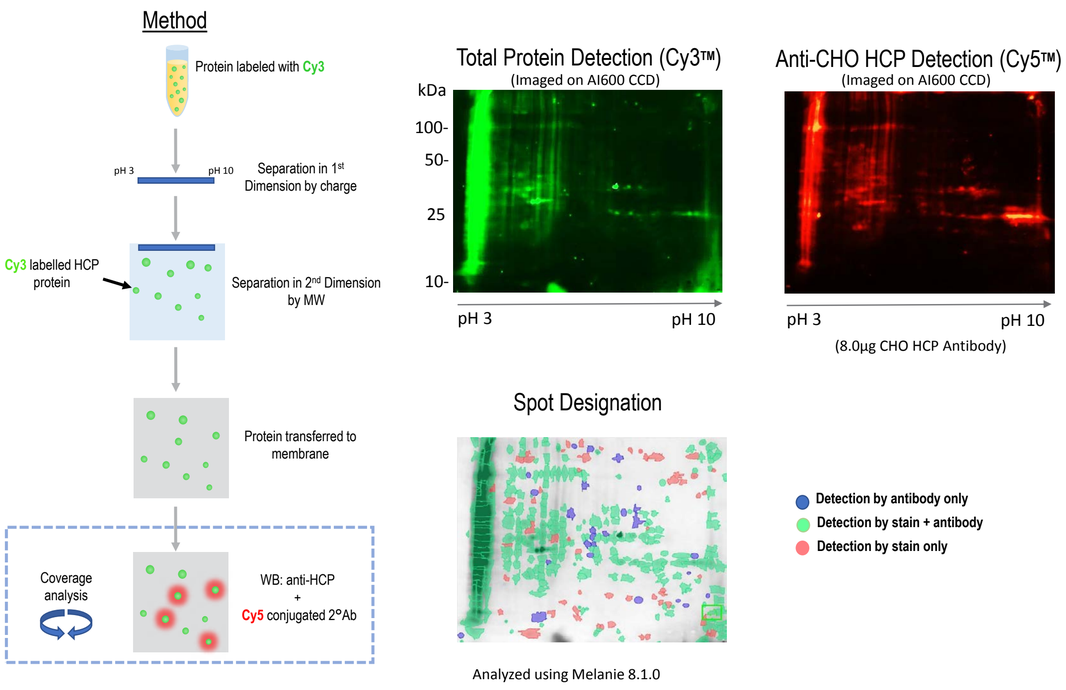
Antibody development



HCP antibody development entails immunization using a representative sample of the HCP extract. The selection of this sample is critical, and can be taken from various stages of the bioprocess workflow. Generation of HCP antibodies requires the use of a variety of immunization protocols to obtain acceptable detection of low abundant or poorly immunogenic proteins. Quality of the antibody is validated by 1D and 2D-SDS-PAGE for immunocoverage in addition to ELISA based assays.

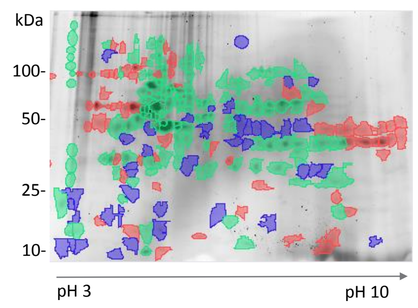
Here we analyze the coverage of a generic anti-CHO HCP polyclonal antibody (Rockland Inc.) as an alternative to process specific reagents that are functional for HCP detection in 2D WB and ELISA.

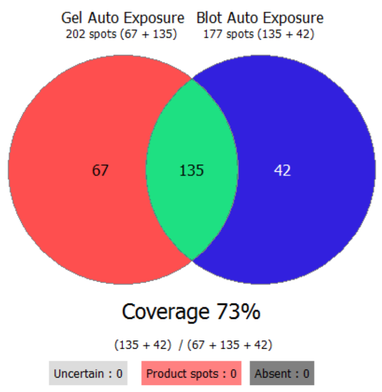
2D-DIBE coverage detection



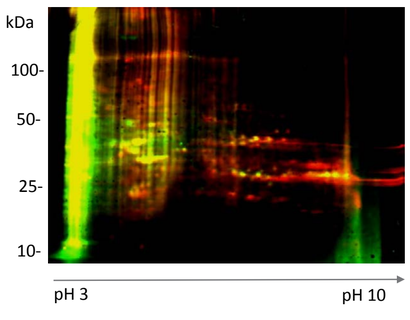
Comparative Results & Conclusions

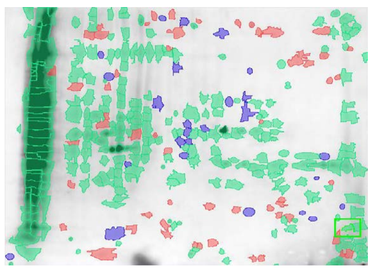
2D-Chemi Coverage Results

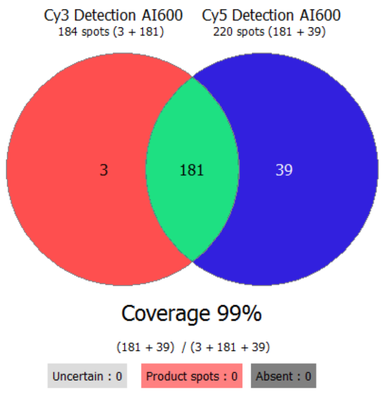




2D-DIBE Coverage Results







Anti-CHO HCP antibody coverage:

- Sensitivity and immunoreactivity of the antibody is robust for proteins in all 4 quadrants of the 2D protein map (data not shown).
- Difficult to detect proteins in the lower molecular weight regions are identified by the CHO HCP-antibody presented here.
- Low concentrations of anti-CHO HCP antibody are sufficient to obtain good signal in both assays.

Chemiluminescence assay:

- Parallel detection of immunoreactive high molecular weight proteins and low abundant low molecular weight proteins requires imaging of the WB at several exposure times. Shown here, a single exposure time was analyzed. Multiple exposure times (often 3-4 exposures) need to be evaluated to determine accurate coverage.
- Detection of high molecular weight protein in overexposed regions of the blot can be challenging with chemiluminescence.

2D-DIBE assay:

- Bypasses the need of the alignment step (gel-WB) in coverage analysis.
- Simultaneous visualization of total protein and antibody coverage allows for overall accuracy in spot alignment and reduces user error in the analysis step.
- Well-defined, resolved spots in 2D-DIBE make manual spot review and designation easy, quick and free of user bias.
- 2D-DIBE assay allows for an approximate 10-fold increase in sensitivity compared to the chemiluminescent assay.

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Robust HCP Coverage Analysis with Dedicated Melanie Software

S. Voordijk[1], M. Sayeed[2], G. Bouchet[3], S. Catherinet[3], D. Walther[3], P. Beckett[4], I. Xenarios[3], J. Hirano[4], K. Abarca Heidemann[2]

[1]Geneva Bioinformatics (GeneBio) SA, Geneva, Switzerland; [2]Rockland Immunochemicals Inc., Limerick, PA 19646, USA;
[3]SIB Swiss Institute of Bioinformatics, Geneva, Switzerland; [4]GE Healthcare Life Sciences, Uppsala, Sweden

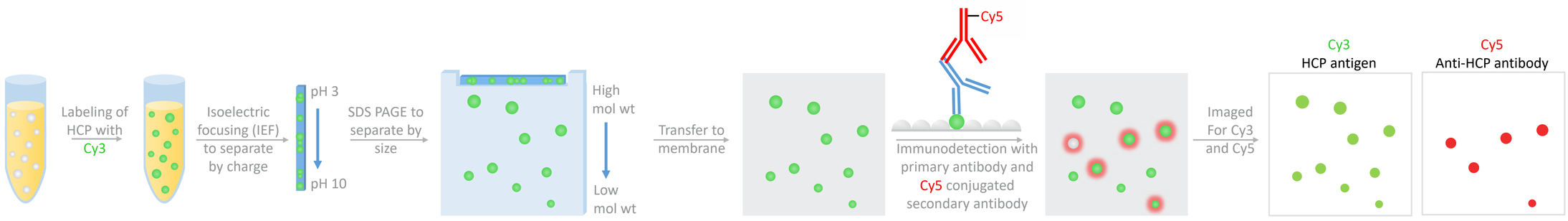
Introduction

Host Cell Protein (HCP) impurities must be carefully identified, minimized and monitored throughout development and manufacturing of biopharmaceutical products to guarantee patient safety and drug efficacy. While HCP ELISA is a critical component of HCP contaminant detection, regulatory agencies require demonstration that the polyclonal antibody mixture used in the ELISA is broadly reactive against a wide range of potential HCPs.

During the development and validation of antibodies, 2D gel electrophoresis followed by Western blotting is a standard approach to determine coverage of the HCP specific antibody, i.e. the percentage of immunodetection the antibody offers for the total population of HCP. Yet, vastly different spot patterns are often seen between independent gels and blots, as well as subjective and time-consuming image analysis with unsuitable tools have presented serious challenges to coverage assessment.

Here we show how the application of 2D Differential in Blot Electrophoresis (2D-DIBE) for the characterization of an anti-CHO cell antibody removes the need for subjective and laborious blot-to-gel matching. Image analysis with the dedicated Melanie™ Coverage software further reduces subjectivity and analysis time.

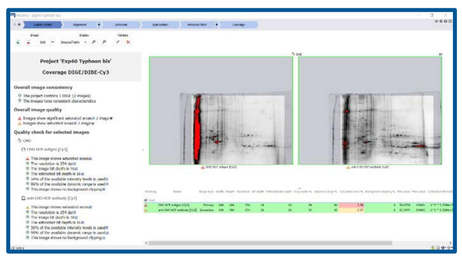
2D Differential in Blot Electrophoresis (2D-DIBE)



Methods

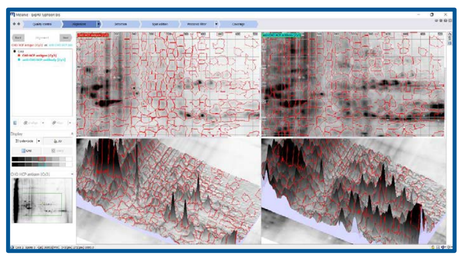
CHO-HCP sample was pre-labeled with Cy3™ and separated on a 2D gel. Protein was transferred from the gel to a LF-PVDF membrane. Blot was incubated with a generic anti-CHO-HCP polyclonal antibody (Rockland Inc.) and a Cy5™ pre-labeled secondary antibody. Blot images were acquired with Amersham™ Typhoon™ at 100 µm resolution, saved as 16 bit images and analyzed with Melanie Coverage software, using the dedicated workflow and settings below.

Image Analysis Workflow



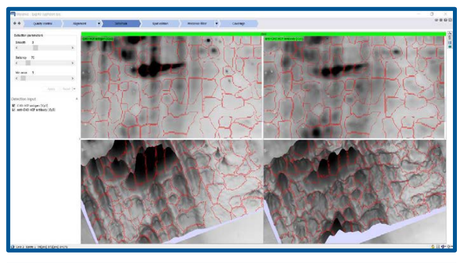
Quality control

A coverage DIGE/DIBE project was created and images imported. Cy3 was indicated as dye for the primary image (useful when analyzing replicate DIBE pairs). Automatic image quality checks were applied. Images were cropped and the contrast adjusted.



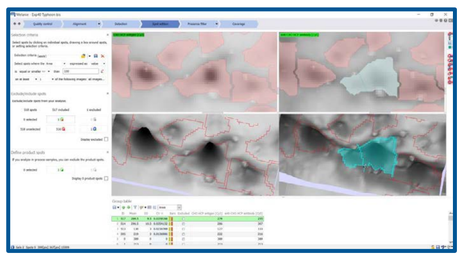
Alignment

As the 2D-DIBE images are from the same blot, no alignment was necessary. For alignment of replicate DIBE pairs, dedicated tools allow systematic review of alignment pairs (only primary images need be aligned). Where necessary, automatic or user matches can be edited in 2D and 3D views.



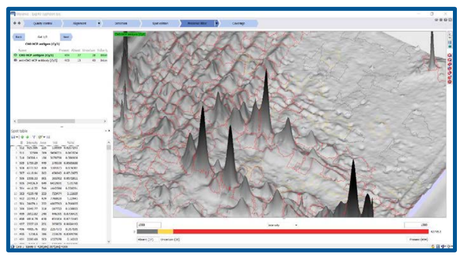
Detection

Spot detection parameters were fine-tuned (Smooth=3, Saliency=70, Min Area=5) and both images were selected to determine the single spot map. 513 spots were automatically detected.



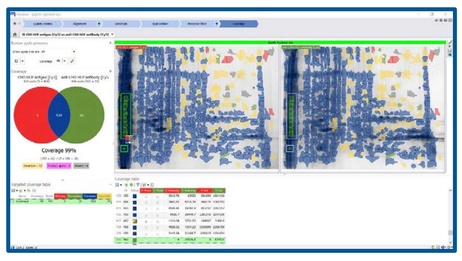
Spot edition

No spots were deleted or excluded. A few spots were added or edited (split, grow, shrink) in the 2D or 3D views while having both images visualized to reduce bias. A total of 520 spots were selected for further review.



Presence filter

For each image, a three level spot filter was applied to categorize spots as absent, uncertain, or present. Intensity was selected for filtering, and the two thresholds applied were 1000/1300 for CHO-HCP antigen and 2000/3500 for anti-CHO-HCP antibody.

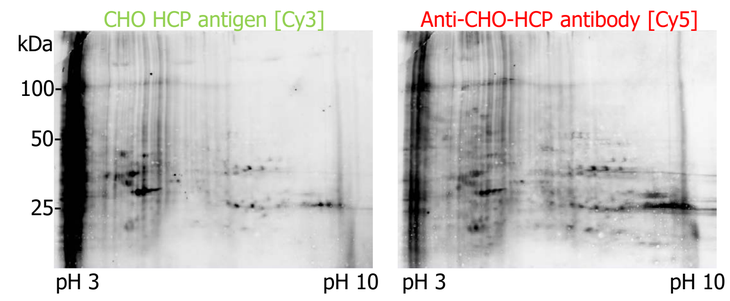


Coverage

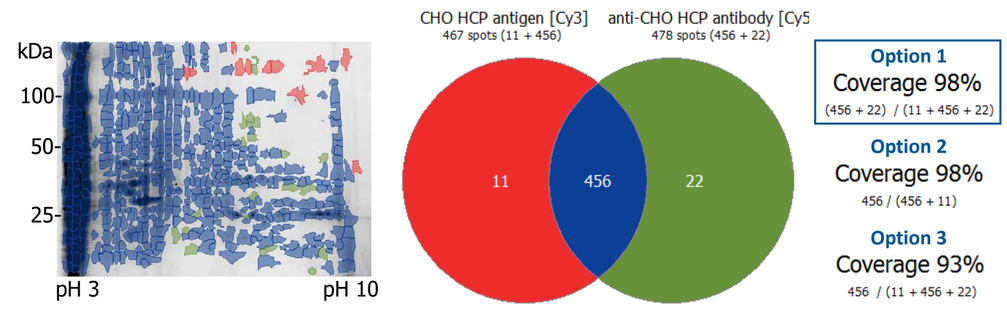
The 74 uncertain spots were quickly reviewed using the 3D view and table to confirm appropriate coverage status (presence or absence on primary image, secondary image, or both). % coverage and Venn diagram were exported after completed review. Total analysis took less than an hour.

Results and Conclusions

Raw images



Coverage results

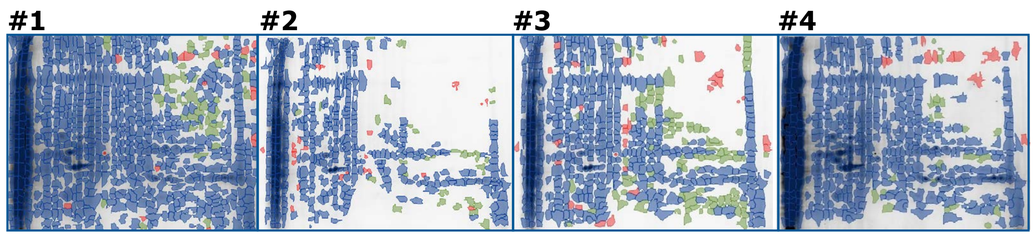


The anti-CHO-HCP antibody presented here shows high immunoreactivity with a coverage of 98% as calculated with the formula in Option 1 (setting in the software). Coverage is even 100% for spots with mol wt < 20 kDa (targeted coverage results not presented here).

Reproducibility

Images were analyzed by 4 different operators from 3 different affiliations, with varying experience with the Melanie Coverage software. All users reported that the analysis took less than an hour. Mean coverage was 96%, with a CV of 2.1%.

Analysis of 2D-DIBE data with Melanie therefore provides reliable, reproducible, unbiased, and fast coverage results to validate antibody reagents.



User	Coverage (%)	Spots	Primary	Secondary	Common
#1	98	764	18	43	703
#2	95	481	23	32	426
#3	94	572	34	101	437
#4	98	489	11	22	456

References

- Amersham Typhoon (www.gelifesciences.com/typhoon), GE Healthcare Life Sciences, Uppsala, Sweden
- Melanie Coverage software (www.2d-gel-analysis.com), developed by SIB Swiss Institute of Bioinformatics, Geneva, Switzerland and available from GE Healthcare Life Sciences, Uppsala, Sweden and GeneBio, Geneva, Switzerland.
- HCP-antibodies (http://www.rockland-inc.com/Host-Cell-Protein.aspx) developed by Rockland Immunochemicals Inc. Limerick, PA 19646, USA

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