Three novel protocols for protein purification by Free-Flow-Electrophoresis

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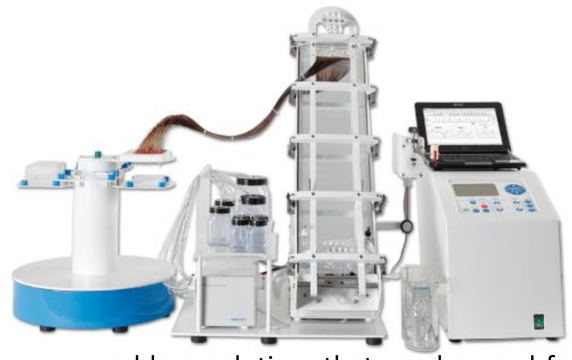


Introduction

Free-flow electrophoresis (FFE) is a **matrix-free** electrophoretic separation technique. FFE is an analogous technique to **capillary electrophoresis**, with a comparable resolution, that can be used for scientific questions, were semi-preparative and **preparative amounts** of separated samples are needed. It is used to quantitatively separate samples according to differences in charge or isoelectric point. Because of the versatility of the technique, a wide range of protocols for the separation of samples like rare metal ions, protein isoforms, multiprotein complexes, peptides, cells, DNA origami, blood serum and nanoparticles exist.

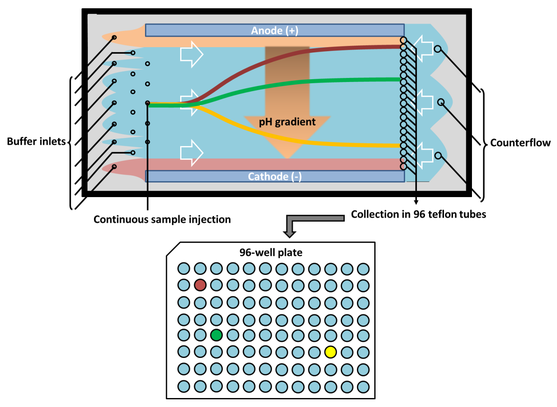
Many clinically and scientifically relevant proteins undergo posttranslational modification (PTM) stemming for example from chemical adducts (glycosylation, phosphorylation, etc.) or mRNA splicing. These protein isoforms can differ from each other in their activity and therefore need to be closely studied. For therapeutic and clinical applications, it can be beneficial and/or necessary to separate isoforms from each other ahead of clinical application. **Monoclonal antibodies (mABs)** are a good example of such proteins, often bearing alternative and closely related PTMs that make the mAB isoforms challenging to separate. However separation can be important for characterization and ultimately for commercial production.

Here we present the quantitative separation of a mAB, by utilizing three complementary FFE methods. Additionally we demonstrate the separation of a mAB with a pI of 9.8, by using our proprietary ProLytes system.

IEF-FFE

The first technique relies on a continuous separation of samples with respect to the isoelectric point of each component. To this end a pH gradient is generated by **commercial ampholytes**, which was used for preparative separation of monoclonal antibodies up to 100 mg. The throughput is 3 mg/h with a resolution of < 0.02 Δ-pH.

Principle of IEF Free Flow Electrophoresis



Separation of mAB by FFE-IEF

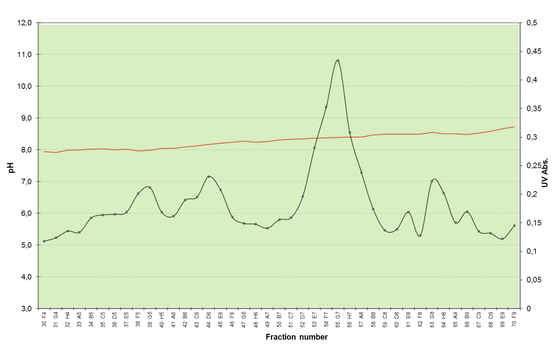


Figure 1: IEF-FFE separation of crude mAB sample on pH with an ultraflat pH-gradient from 8 to 8.5. The fractions within the green area were applied to an IEF gel (Figure 2).

IEF-PAGE

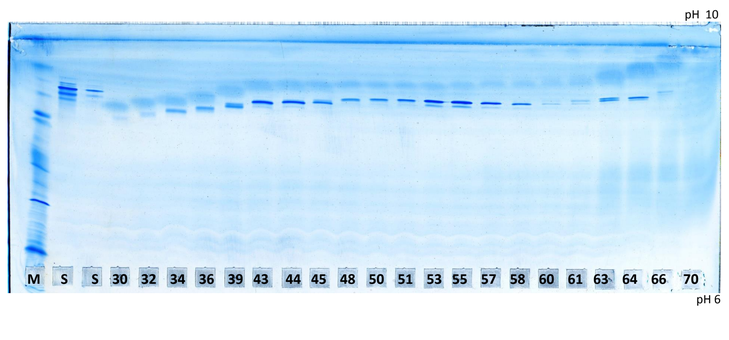


Figure 2: IEF-PAGE (pH 6-10) of the crude sample (S) and selected fractions of IEF-FFE separated mAB sample. M: Serva IEF Marker.

Re-electrophoresis

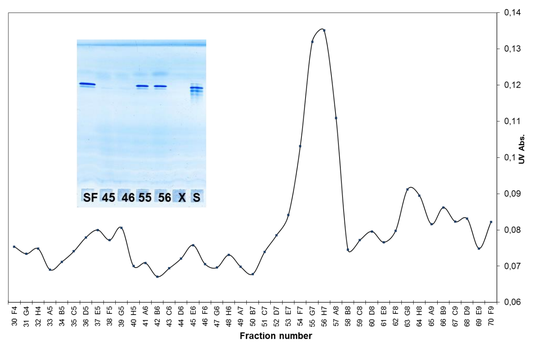


Figure 3: Re-FFE-electrophoresis was performed with fraction 56 to verify the stability of the separation process.

Material and Methods

Protein throughput:	2,5 mg/h (max. 3 mg/h)	Standard buffer flow rate:	33 ml/h	Temperature:	5 °C
Voltage:	2900 V	Transit/Separation time of sample:	20 minutes	Resolution:	0.02 Δ-pH, 0.04 Δ-pI
Field strength:	490 V/cm	pH-gradient:	8-9	IEF-gel:	Serva Focus gel pH 6-11

ProLytes™-FFE

The second technique, matching handling, resolution and throughput of the IEF-FFE, makes use of a proprietary **mixture of acids and bases** to generate the pH-gradient needed for separation. Because no ampholytes get in contact with the samples, clinical application of the separated isoforms is possible.

Separation of mAB1 by ProLytes™-FFE

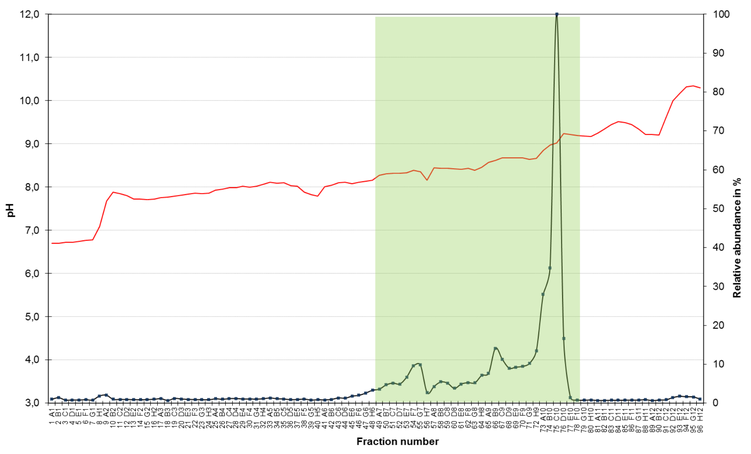


Figure 4: Relative abundance (black line) of the separated mAB sample and pH profile (red line). The fractions within the green area were applied to an IEF gel (Figure 5).

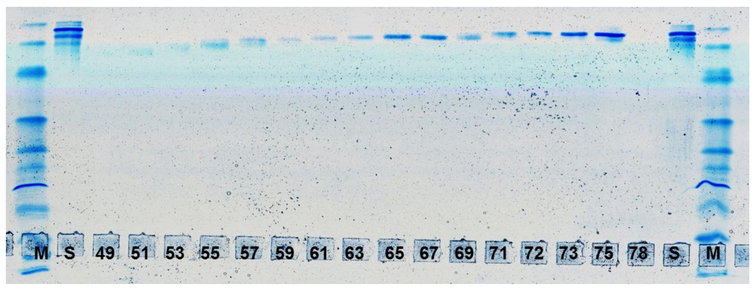


Figure 5: IEF-PAGE (pH 6-10) of the crude sample (S) and selected fractions of ProLytes-FFE separated mAB sample. M: Serva IEF Marker.

Separation of mAB2 (pI = 9.8) by ProLytes™-FFE

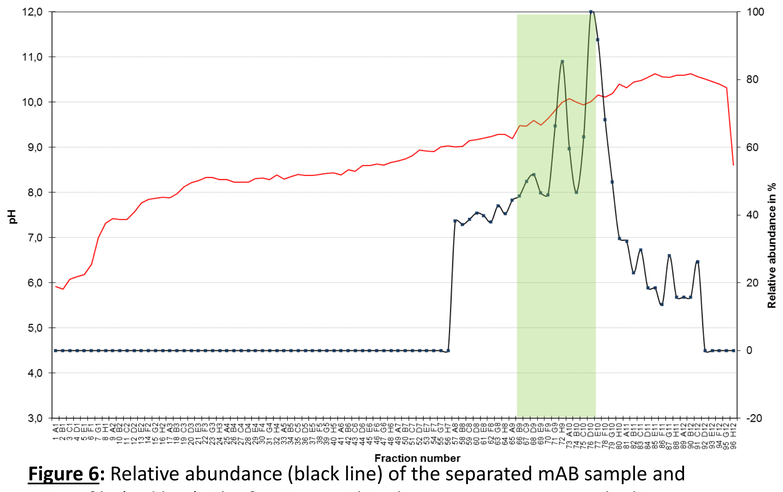


Figure 6: Relative abundance (black line) of the separated mAB sample and pH profile (red line). The fractions within the green area were applied to an IEF gel (Figure 5).

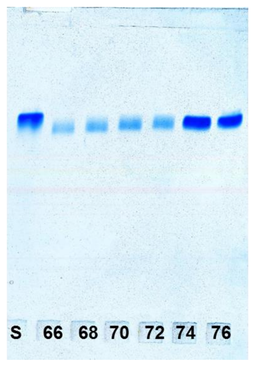


Figure 7: IEF-PAGE (pH 7-11) of the crude sample (S) and selected fractions of ProLytes-FFE separated mAB sample.

Protein isoform separation using FFE:

- **Entirely liquid** methods (no gels, no matrix)
- **Native conditions** maintain structure and function of separated isoforms
- **Quick and easy** to set up and operate
- **High resolution** separations
- Scale **from analytical to milligram** preparation ability
- One instrument supports **many different separation needs**:
  - Flexible and adaptable methods
  - From simple to highly complex samples
  - Specific protein targets (and their unique characteristics)
  - From basic research to production-scale throughput

IEF-FFE:

- The method of choice for
  - Characterizing complex mixtures
  - Screening preparations of unknown isoelectric composition
- Uses ampholyte cocktails
  - Commercially available in a variety of defined pH ranges, from broad to narrow
- Unique continuous run method (not batch) can allow high separation capacity of up to 100 mg of mAB
- Resolution of < 0.02 Δ-pH.

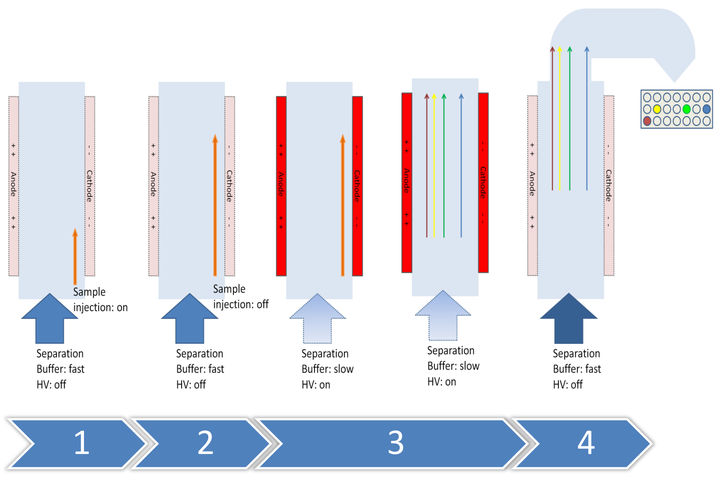
ProLyte-FFE:

- Same workflow as IEF-FFE
- Unique benefits versus IEF-FFE
  - No dependency on commercial ampholytes
  - No change in quality between batches
  - Clinical application possible
  - Easy removal of separation buffer
  - Custom pH-gradients possible
  - Custom pH-range possible
  - Wide pH range

IZE-FFE

The third technique (IZE-FFE) relies on differences of electromigration in a stepwise pH-gradient, formed by the use of different acid and base containing buffers over the width of the separation chamber. Because the proteins are not separated at their isoelectric point, this technique minimizes protein-protein interactions and its short interval time of 10 minutes and throughput of up to 100 µg protein per interval, allows for rapid analysis or preparation of samples by continuous collection of fractions. Direct MS measurements are possible, due to the lack of ampholytes and polymers.

Principle of IZE Free Flow Electrophoresis



Separation of mAB by FFE-IZE

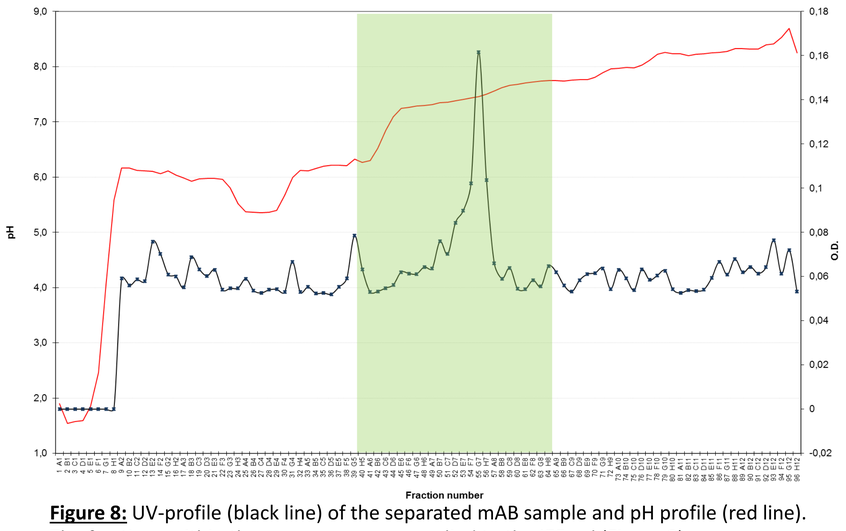


Figure 8: UV-profile (black line) of the separated mAB sample and pH profile (red line). The fractions within the green area were applied to the IEF gel (Figure 8).

IEF-PAGE

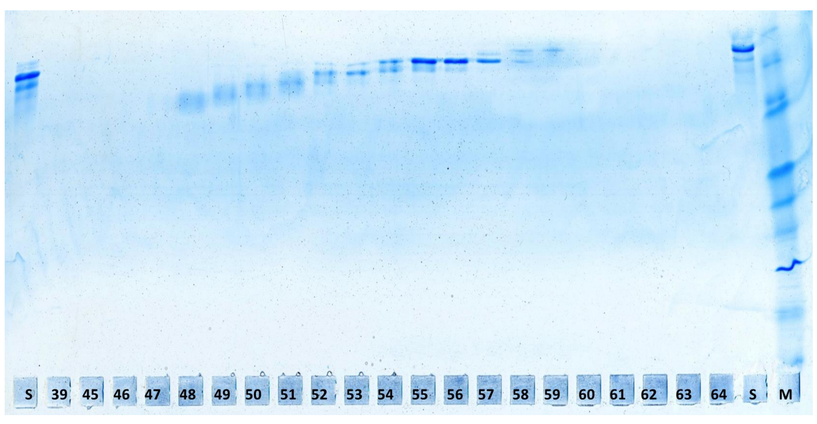


Figure 9: IEF-PAGE of the crude sample (S) and selected fractions of IZE FFE separated mAB sample.

The Antibody samples for this study were supplied by Biogen, RTP, North Carolina, USA.