

# Imaged cIEF – The best solution for complex biomolecules

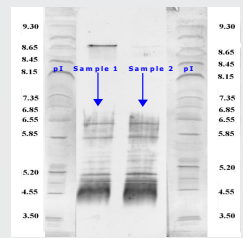


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## Introduction

The pI of a protein is usually assessed to identify a molecule in the frame of process development, characterization, comparability, release and stability studies. Conventionally the most common analytical technique used to determine the pI is based on IEF gel which separates proteins according to the charge (determined by the number of acidic and basic residues in the protein). The present work provides a case study where the molecule attributes represent an obstacle to achieve acceptable results with traditional IEF gel. Different approaches were evaluated in order to find optimal conditions for the identification of the isoforms profile of the molecule under analysis. Trials including different gels, sample treatments, and running conditions did not improve the performance of the analysis. In contrast to other IEF methods developed for several other products, the gel-based method did not yield a satisfactory performance, neither in terms of isoform separation nor in reproducibility. A typical IEF gel is shown in the next figure.

**Figure 1 IEF analysis of the highly glycosylated protein under investigation**



This unsatisfactory result is attributable to the complexity of the molecule itself, i.e. a relatively large size, high degree of glycosylation (N- and O-linked), sialylation, and phosphorylation.

The data presented demonstrate that the Imaged Capillary Electrophoresis (ICE) by iCE280 represents a powerful alternative technique to conventional gels because it offers a more rapid separation, higher resolution and satisfactory reproducibility.

## Experimental conditions

- Equipment**
- CE system:** iCE280 Analyzer (Convergent Biosciences)
- cIEF cartridge:** 50 mm length, 100 µm ID column, Restek RTX200 coating
- Reagents and analytical conditions**
- Electrolytes:** Anolyte- 80 mM H3PO4; catholyte- 100 mM NaOH
- Carrier Ampholytes:** 2% pH 3.5-5.0 Ampholine, 2% pH 2.5-5.0 Pharmalyte
- Sample additives:** 0.35% methyl cellulose, 4 M urea
- Internal pI Marker:** pI 3.59, pI 4.65
- Focusing Time:** 1 + 12 minutes
- Focusing Voltage:** 1 minute at 1500V (300 V/cm) and 12 minutes at 3000V (600 V/cm)
- Protein sample**

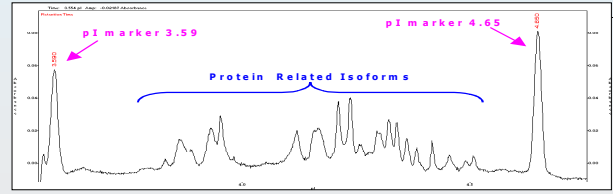
Highly purified and concentrated samples were added with Ampholyte samples solution (MC 1%) Urea 8M + Pharmalyte 2.5-5.0 + Ampholine 3.5-5.0 + pI 3.59 + pI 4.65 + purified Water then applied to the capillary by pressure rinse mode (2000 mBar) for 160 sec.

## Results and Discussion

Despite a large number of experiments and operative conditions tested, traditional CZE and cIEF were found to be not suitable for the analysis of the protein object of the study. On the other hand the data generated using the new technology, iCE280, were satisfactory both in terms of isoforms resolution and consistency.

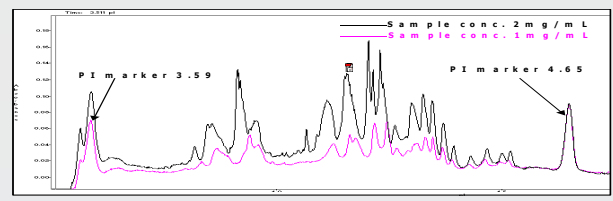
The Figure below shows a typical example of the result obtained with the application of the final method.

**Figure 2 Separation of protein isoforms**



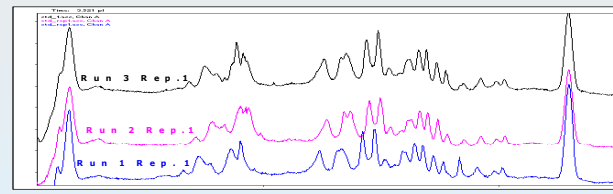
The analysis of the protein sample samples revealed that the molecule is highly acidic and distributed in several isoforms in the pI range between pH 3.59 and 4.65. Figure 3 shows that the sample concentration (1-2 mg/mL) does not influence the isoforms separation.

**Figure 3 Resolution of isoforms in samples at 1-2 mg/ml concentration**

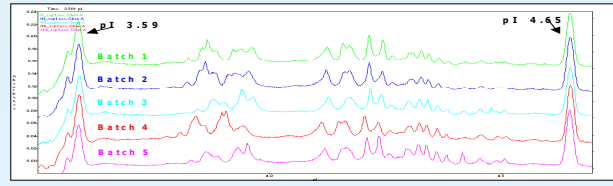


To ensure the applicability of the methodology for routine QC test as support to process development and release, the performance of the method was demonstrated through independent experiments carried out on the same sample (see figure 4) and on purified protein prepared by different USP-DSP runs (see figure 5).

**Figure 4 Sample analysed in 3 independent analytical runs**



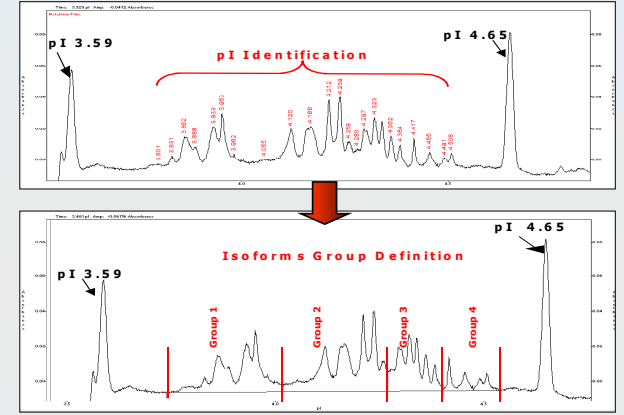
**Figure 5 Purified samples from independent USP/DSP runs**



## Quantitative approach

The complex isoform pattern of the protein under study required the development of a robust approach for the quantification of the isoforms in order to use the test as a release and for supporting production activities related to the establishment of the process consistency. Figure 6 shows the approach which was applied to perform the reproducibility study.

**Figure 6 Identification of pI and assessment of the integration**



**Table 1 Quantification approach for isoform group in complex biomolecules**

Isoforms group	pI range	Run	% area (Average 3 replicates)
1	3.80 ÷ 3.98	1 (rep. 1-3)	28.8
		2 (rep. 1-3)	31.29
		3 (rep. 1-3)	30.32
2	4.05 ÷ 4.26	1 (rep. 1-3)	44.94
		2 (rep. 1-3)	43.06
		3 (rep. 1-3)	42.39
3	4.28 ÷ 4.38	1 (rep. 1-3)	21.14
		2 (rep. 1-3)	21.56
		3 (rep. 1-3)	21.65
4	4.42 ÷ 4.51	1 (rep. 1-3)	5.12
		2 (rep. 1-3)	4.09
		3 (rep. 1-3)	5.64

## Conclusions

The results obtained from the study presented have demonstrated a superior performance of the Imaged Capillary Electrophoresis in testing heterogeneous and complex biomolecules.

- The rapid analysis facilitates the execution of routine QC work.
- The high reproducibility guarantees the use of the test for supporting process development and improvement.
- The quantitative approach allows the use of the methodology for pI monitoring process robustness.

## Acknowledgements

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