

# Capillary Isoelectric Focusing Electrophoresis Method Development for Bone Morphogenetic Protein 7

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## ABSTRACT

A capillary isoelectric focusing (cIEF) method was developed for analysis of Bone Morphogenetic Protein 7 (BMP-7). The protein has low solubility at neutral pH. To achieve an interpretable cIEF result, the sample required solubilization in 6 M urea and 100 mM DTT and incubation at 37°C for 10 minutes for the complete reduction. Using a combination of Pharmalyte pH 8-10.5 with Pharmalyte pH 3-10 extended the separation range to the pI over 10. Maintaining the autosampler temperature at 28°C during the cIEF analysis resulted in the best reproducibility of the assay. Finally, the focusing time of 40 minutes was required to obtain good separation and a clear baseline.

## INTRODUCTION

Isoelectric focusing (IEF) is a high-resolution separation technique used in protein and peptide analysis. Capillary isoelectric focusing (cIEF) requires only a small amount of sample (10 µg per injection) and relatively short running time (10 to 40 minutes for most samples). Because the technique can be operated automatically, it produces improved reproducibility comparing to the traditional gel IEF.

BMP-7 is a glycosylated dimeric protein with multiple isoforms. The sources of the heterogeneity contributing to the isoforms are N-terminal truncations, glycosylation, and other post-translational modifications. In addition, BMP-7 is a very hydrophobic protein and relatively insoluble in aqueous media at neutral pH, which add challenges for developing the cIEF method. To overcome the relative insolubility of the protein, BMP-7 must be denatured and reduced prior to cIEF analysis. The cIEF will be used to characterize the protein and to monitor the changes in the protein isoforms by detecting pI shift and components distribution changes.

## METHODS

### cIEF Method

**Instrument:** The cIEF system, purchased from Convergent Bioscience Ltd, Toronto, Canada, includes an iCE280 analyzer, an Autosampler and a syringe pump. Samples were loaded through the Autosampler switch valve and iCE280 Analyzer's switch valve into a 50 mm - long, transparent capillary column. The 80 mM phosphoric acid solution and the 100 mM sodium hydroxide solution were used as anolyte and catholyte. When a consistent voltage was applied, a pH gradient was formed across the capillary.

**Sample Preparation:** Samples were denatured and reduced in 0.35% methyl cellulose containing urea and 1,4-dithiothreitol (DTT), then mixed with carrier ampholytes (Pharmalyte®, Sigma).

**cIEF Analysis:** The sample injection volume was 35 µl. A one-minute pre-focusing was performed at 1500 V and a 10 to 40 - minute focusing was performed at 3000 V. The separation profile was detected at 280 nm.

### Gel IEF Method

Samples were dissolved in sample buffer composed of 9 M urea, 2% CHAPS, 100 mM DTT and 2.8% ampholyte pH 3-10 as carrier ampholyte. The denaturation and reduction was performed at ambient temperature for 2-3 hours. The gel contained 2% agarose, 9 M urea, 2% CHAPS and a mixture of Servalyt 5-9 and Servalyt 9-11. The gels were focused at 1.25 W per gel for 5-6 hours.

## RESULTS

### Optimization of Urea Concentration

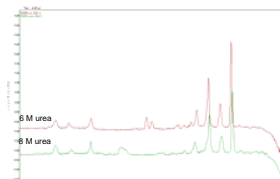


Figure 1. Samples were denatured in 6 M urea or 8 M urea. Samples were reduced with 100 mM DTT by incubation at 37°C for 10 minutes. 8% Pharmalyte pH 3-10 was used as carrier ampholytes. Focusing was performed at 3000 V for 10 minutes. Results show that 6 M urea produced the better focusing result than 8 M urea did.

### Optimization of DTT Concentration and Reduction Time

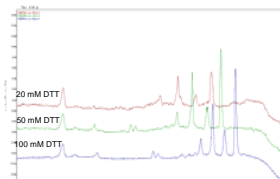


Figure 2. Samples were denatured in 6 M urea. DTT concentrations were 20 mM, 50 mM and 100 mM. Reduction was done by incubation at 37°C for 10 minutes. 8% Pharmalyte pH 3-10 was used as carrier ampholytes. Focusing was performed at 3000 V for 10 minutes. Results show that 20 mM and 50 mM DTT were not sufficient for the complete reduction.

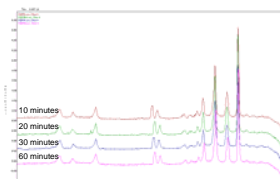


Figure 3. Samples were denatured and reduced with 6 M urea and 100 mM DTT. Reduction was performed at 37°C for 10 minutes, 20 minutes, 30 minutes or 60 minutes. 8% Pharmalyte pH 3-10 was used as carrier ampholytes. Focusing was performed at 3000 V for 10 minutes. The results demonstrate that no difference was made when reduction time was ranging from 10 minutes to 60 minutes.

### Effect of Sample Concentration

Table 1. Repeatability of Main Peak pI at Different Sample Concentration (% CV)

Sample Conc.	Peak 6	Peak 7	Peak 8	Peak 9
0.25 mg/mL	0.05	0.03	0.01	0.01
0.35 mg/mL	0.07	0.03	0.04	0.03
0.50 mg/mL	0.04	0.05	0.03	0.03

Table 2. Repeatability of Main Peak Area % at Different Sample Concentration (%CV)

Sample Conc.	Peak 6	Peak 7	Peak 8	Peak 9
0.25 mg/mL	2.99	1.87	1.19	2.88
0.35 mg/mL	2.33	0.91	0.45	0.74
0.50 mg/mL	0.64	0.43	0.93	0.71

The % CV was calculated from the results of three repeats of each sample at different concentration. The data indicates that sample concentration, within the range 0.25 – 0.5 mg/mL, had no impact on the repeatability of the pI results. Whereas, the % CV of the peak area percentage was significantly smaller when the sample concentration was 0.5 mg/mL than that when the sample concentration was 0.25 mg/mL, i.e. the higher sample concentration gave the better reproducibility.

### Effect of Autosampler Temperature

Table 3. Repeatability of Main Peak pI at Different Autosampler Temperature (% CV)

Temperature	Peak 6	Peak 7	Peak 8	Peak 9
20 °C	1.72	1.76	1.87	1.89
28 °C	0.08	0.05	0.06	0.06

Table 4. Repeatability of Main Peak Area % at Different Autosampler Temperature (% CV)

Temperature	Peak 6	Peak 7	Peak 8	Peak 9
20 °C	7.7	2.7	2.8	1.5
28 °C	2.2	0.6	0.8	1.2

The samples were analyzed in triplicate. The result shows that the higher temperature benefited the repeatability of the analysis results.

### Optimization of the Focusing Time and the Use of Carrier Ampholytes

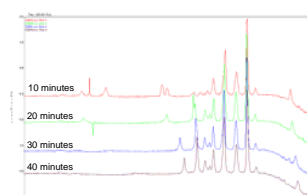


Figure 4. Samples were denatured and reduced with 6 M urea and 100 mM DTT by incubation at 37°C for 10 minutes. 8% Pharmalyte pH 3-10 was used as carrier ampholytes.

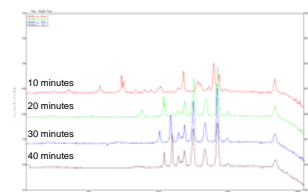
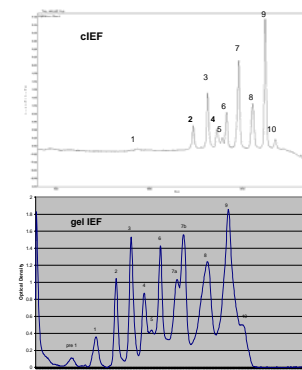


Figure 5. Samples were denatured and reduced with 6 M urea and 100 mM DTT by incubation at 37°C for 10 minutes. Mixture of 4% Pharmalyte pH 3-10 and 4% Pharmalyte pH 8-10.5 was used as carrier ampholytes.

The best separation was obtained at focusing time of 40 minutes and with 4% Pharmalyte pH 3-10 and 4% Pharmalyte pH 8-10.5 as the carrier ampholytes.

### Comparison of cIEF Profile vs. Gel IEF Profile



## CONCLUSION

A cIEF method has been developed for analysis of BMP-7. The results of the optimization experiments suggest that the denaturation and reduction of the sample need to be done in 0.35% methyl cellulose containing 6 M urea and 100 mM DTT by incubation at 37°C for 10 minutes. The combination of 4% Pharmalyte pH 8-10.5 with 4% Pharmalyte pH 3-10 give a pH range covering all the isoforms of the protein. A one-minute pre-focusing at 1500 V and 40 minutes focusing at 3000 V are necessary to complete the separation of isoforms of the protein. The IEF profile from the cIEF method was similar to the profile from traditional gel IEF, which suggests that the cIEF method is accurate and may be used to replace the traditional gel IEF method.

## ACKNOWLEDGEMENTS

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