

Comparison of Three IEF Methods for the Analysis of a BiTE® Antibody

Jennifer Duehring*, Christopher Kober, James Stattel, Robert Strouse

MedImmune, Analytical Biochemistry Department, Gaithersburg, MD 20878 USA

ABSTRACT

Isoelectric focusing (IEF) is a commonly used technique for determining the heterogeneity and stability of protein therapeutics. The densitometric profiles and pIs of BiTE antibody isoforms were determined using three different IEF methods. These methods were: 1) a fluorescent-stained IEF slab gel; 2) silver-stained IEF slab gel; and 3) an imaged capillary IEF method. The data was evaluated to determine the precision, accuracy and reproducibility of each method. Compared to the slab-gel based methods, the imaged cIEF test proved to be more accurate and precise. The imaged cIEF test was more reproducible when the assays were performed on different days and by different analysts. Furthermore, analyses of accelerated stability samples demonstrated that the imaged cIEF test was the superior stability indicating method for this BiTE antibody.

INTRODUCTION

Isoelectric focusing (IEF) is an analytical tool used to determine the isoelectric point of proteins based on their electrophoretic mobility. This technology is based on the observation that in a pH gradient under the influence of an electric current, a protein will migrate (or "focus") to its specific isoelectric point (pI) where its net charge becomes zero. IEF determinations are traditionally performed using slab gels. However, these methods are generally only semi-quantitative in nature.

BiTE antibodies are bi-specific T cell engager molecules. The BiTE antibody analyzed in this study has anti-CD19 and anti-CD3 moieties at opposite ends of the molecule and works by recruiting CD3-expressing T cells to target/destroy CD19-expressing cells. Two Novex slab-gel based IEF methods, each using a different detection reagent, are currently used at MedImmune to determine the pI values of the T cell engaging BiTE antibody but both methods have limitations. The fluorescence method relies solely on the analyst's visual inspection, making it a subjective and qualitative assay, while the silver stain method is unreliable due to the variability in protein visualization inherent in silver-stained gels.

The Convergent ICE280 Analyzer is a capillary-based IEF (cIEF) system that holds many advantages to IEF analyses performed using slab gels. The ICE280 Analyzer incorporates the advantages of column based separation technology with higher focusing voltage and minimal focusing time, resulting in a reliable automated system for quantifying IEF results.

This presentation describes the comparison of the ICE280 method to both the fluorescent and silver stained slab gel IEF methods with respect to accuracy and precision for the characterization of a BiTE antibody.

MATERIALS AND METHODS

Fluorescent-stained IEF Slab Gel Method: Samples were diluted 1:1 in pH 3-10 2X Sample Buffer, incubated at ambient temperature for 10 minutes, and then loaded onto an Invitrogen Novex IEF gel [pH gradient 3-10 (0.5 µg protein/lane)]. The gel was focused at 100 V / 5 mA / 2 W for 1 hour, then 200 V / 5 mA / 2 W for 1 hour, followed by 500 V / 6 mA / 3 W for 1 hour using gentle agitation. Following focusing, the gel was fixed in 12% trichloroacetic acid, destained, and fluorescent stained overnight with Sypro Ruby protein gel fluorescence stain. Gels were imaged on the BioRad VersaDoc instrument.

Silver Stained IEF Slab Gel Method: Samples were concentrated using Microcon 10,000 molecular weight cutoff filters in Formulation Buffer, diluted 1:1 in 4% pH 3.5-10 Ampholine solution, and incubated at ambient temperature for 10 minutes. The pH 3.5-10 gel (GE Healthcare) was loaded at 2 µg/lane and focused at 1500 V / 25 mA / 15 W for 1 hour and 45 minutes. Following focusing, the gel was fixed, sensitized, stained, and destained using the GE Healthcare Processor Plus automated staining instrument and PlusOne Protein Silver Staining kit. Gels were imaged on the BioRad GS-800 Calibrated densitometer.

Imaged cIEF Method: Samples were diluted 1:1 with Ultra Pure Water and filtered using Microcon 10,000 molecular weight cutoff filters. Additional Ultra Pure Water was used to wash the filter and the protein was removed from the filter at approximately four times its original concentration. The concentrated BiTE antibody was added to 1% Methylcellulose solution, Pharmalyse pH 3-10, and pI Markers. The samples were loaded onto the ICE280 and focused at 1500 V for 1 minute, followed by 3000 V for 6 minutes. Samples were analyzed using the EZChrom software from Convergent.

RESULTS: Assay Presentation

Table 1. Comparison of Fluorescent, Silver Stain, and ICE280 IEF Methods. Even though the staining portion to the silver stain method is automated, the ICE280 also has the ability for much higher sample throughput with less analyst intervention. Total sample turnaround times for the slab gels were based on maximum capacity runs.

ATTRIBUTE	Fluorescent IEF (gel)	Silver Stain IEF (Gel)	ICE280 (Capillary Cartridge)
Detection	Indirect	Indirect	Direct
Technology acceptance	Acceptable Industry Standard	Acceptable Industry Standard	Arising New Industry Standard
Sample throughput per run	8 samples/24-36 hours	12 samples/24-36 hours	72 samples/24hours
Waste generation per run	1.5 L	6 L	< 0.01 L
Sample Preparation	45 minutes	45 minutes	45 minutes
Run time per Sample	3 hours	1.75 hours	0.3 hours
Post-run processing time	Up to 2 days (staining, destaining, densitometry)	Up to 2 days (staining, destaining, densitometry)	0.25 hours (manual integration)
Turnaround time: 1 sample	2-3 days	2-3 days	1.5 hours

Assay Parameter	Band #	Mean	SD	%CV
Analyst-to-Analyst Precision (n=3)	Band 1	7.85	0.02	0.25
	Band 2	7.74	0.01	0.11
	Band 3	7.49	0.02	0.31
Day-to-Day Precision (n=3)	Band 1	7.84	0.00	0.03
	Band 2	7.73	0.00	0.05
	Band 3	7.47	0.01	0.11
Repeatability (n=6)	Band 1	7.85	0.02	0.23
	Band 2	7.73	0.01	0.07
	Band 3	7.49	0.01	0.16

Table 2. Intermediate and Repeatability Precision of the ICE280 Assay. These studies determined the analyst-to-analyst, day-to-day, and sample-to-sample variability of the ICE280 method. The overall CV between replicates is < 0.31%, well below the acceptance criteria of ≤10%.

Assay Parameter	Band #	Mean	SD	%CV
Analyst-to-Analyst Precision (n=3)	Band 1	7.85	0.08	0.99
	Band 2	7.68	0.09	1.20
	Band 3	7.51	0.10	1.30
Day-to-Day Precision (n=3)	Band 1	7.80	0.04	0.47
	Band 2	7.61	0.04	0.48
	Band 3	7.44	0.04	0.49
Repeatability (n=6)	Band 1	7.81	0.03	0.43
	Band 2	7.62	0.04	0.47
	Band 3	7.44	0.03	0.42

Table 3. Intermediate and Repeatability Precision of the silver stain slab gel IEF assay. These studies determined the analyst-to-analyst, day-to-day, and sample-to-sample variability of the ICE280 method. The overall CV between replicates is < 1.30%, well below the acceptance criteria of ≤10%.

Comparable numerical data cannot be generated from the BiTE antibody fluorescent IEF assay due to pI Marker identification limitations.

Table 4. Accuracy Comparison of pI Between Expert Protein Analysis System (EXPASy) and Different IEF Assay Methods. This study examined the accuracy by determining the % difference between different assay methods as compared to the EXPASy generated pI value. The sample was analyzed in six replicates. The % difference in pI values clearly demonstrates the ICE280 as comparably accurate with a difference of 8.03%, which is below the acceptance criteria of ≤10%.

System	Quantity One/EZChrom Generated pI	EXPASy Generated pI	% Difference
ICE280	7.85	7.22	8.03
Silver Stain IEF	7.81	7.22	7.55
Fluorescence IEF	n/a	7.22	n/a

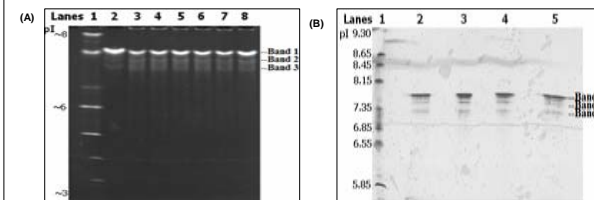


Figure 1. Fluorescence IEF and Silver Stain IEF Gel Images. The BiTE antibody was analyzed by the methods described in the Materials and Methods section. (A) The Fluorescence IEF gel image was digitized using the BioRad VersaDoc Imager powered by the Quantity One software. (B) The Silver Stain IEF gel image was digitized using the BioRad GS-800 Calibrated Densitometer powered by the Quantity One software.

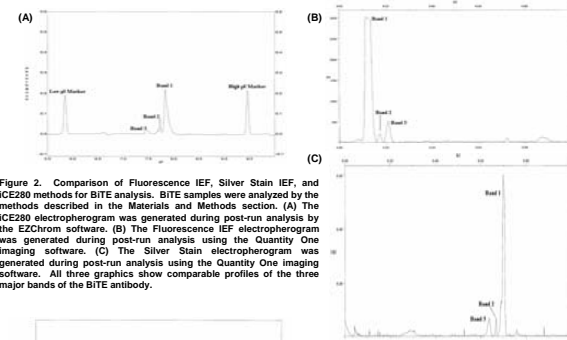


Figure 2. Comparison of Fluorescence IEF, Silver Stain IEF, and ICE280 methods for BiTE analysis. BiTE samples were analyzed by the methods described in the Materials and Methods section. (A) The ICE280 electropherogram was generated during post-run analysis by the EZChrom software. (B) The Fluorescence IEF electropherogram was generated during post-run analysis using the Quantity One imaging software. (C) The Silver Stain electropherogram was generated during post-run analysis using the Quantity One imaging software. All three graphics show comparable profiles of the three major bands of the BiTE antibody.

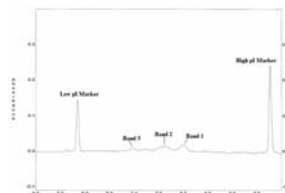


Figure 3. BiTE Photostability Study. The BiTE antibody was placed in a photostability unit for 2 days to promote antibody breakdown. The profile generated by the ICE280 demonstrates the method is stability-indicating because of a reduction in major band peak heights. Compare this to panel A of Figure 2.

CONCLUSION

The ICE280 assay was demonstrated to be an accurate and precise method for IEF determination. Assay accuracy was established through comparisons of the estimated pI from densitometry with the theoretical pI values, while assay precision was demonstrated for repeatability, analyst-to-analyst, and day-to-day experiments. In addition, the ICE280 is capable of generating numerical data, whereas the current IEF methods are more subjective/qualitative.

In addition to the direct comparability of the ICE280 with Fluorescence IEF and Silver Stain IEF results, the ICE280 has significant advantages over these methods. These include reduced sample turnaround times, reduced liquid waste generation, and increased sample throughput. Large gains in laboratory productivity and decreased time to obtain results make the ICE280 a valuable tool in the analytical laboratory.

ACKNOWLEDGEMENTS

We would like to thank Drs. Mark Schenerman and Gail Folea-Wasserman for their support of this work.

