

Abstract: The iCE280 Analyzer, a Capillary Isoelectric Focusing (cIEF) system from Convergent Bioscience, is a relatively new tool for the charge based (pI based) separations of proteins, peptides, and/or other contaminants. The technology can also be used for the determination of isoelectric point (pI) and/or area% of the peaks. The cIEF method has been developed for the analysis of a 70kDa recombinant protein and is also routinely used for the analysis of GSK monoclonal antibody products. Each protein has its own unique isoelectric point where the net charge is zero. The application of voltage to a sample mixed with ampholytes & additives leads to the formation of a pH gradient and causes migration of each charged species to their corresponding pI. Unlike conventional method where it is necessary to mobilize the focused protein bands for detection, the iCE280 module utilizes a whole column imaging capture of the entire separation, being equipped with UV deuterium lamp, a Camera & a CCD sensor.

With this advantage the system is being utilized to enable fast and reliable Analytical Methods development for the separation of charged species. Among the samples/investigations performed with this instrument were: a.) decrease in binding activity of certain mAb products in the Biacore assay which correlated with the presence of increased deamidation products and high pI variant, b.) support of cell line selection, c.) characterization of several monoclonal antibodies, including forced deamidation & variant/s separated by ion exchange chromatography, d.) support of the downstream process development, e.) identification and characterization of the N-term.extension variant of the 70kDa protein and f.) release and stability testing of the final product.

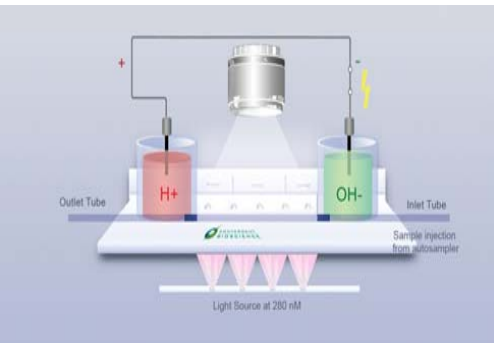
Introduction

Imaged iCE280 Analyzer is a relatively new analytical technique for the determination of isoelectric point (pI) and quantitative analysis of proteins, peptides, their variants and/or contaminants. The imaged Capillary Electrophoresis System (iCE280) utilizes a whole-column UV Imaging detection system with a deuterium lamp as light source (Fig.1). The schematic of the whole column imaging and capture by a digital camera are depicted in Fig.2. Data acquisition & control are performed by the iCE280 instrument. Data processing performed using Empower or EZChrom 6.8 software.



Fig.1

Fig.2



Materials & Methods

Capillary Isoelectric Focusing method (cIEF): Acquisition and analysis were performed on an iCE280 Convergent Bioscience system equipped with CFR Software Version 2.3, cIEF cartridge-FC coating (convergent # 101701), an Alcott 719L autosampler, and a Maximizer Lab Alliance pump for continuous delivery of methylcellulose solution. Calibration of pI markers were performed by the ICE software followed by conversion and processing of data by Empower or EzChrom version 6.8. Reagents used for sample preparation were 10MUREA solution, dithiothreitol (DTT), HPLC grade water, 5-8 Pharymalte from Sigma, and synthetic pI markers special order from Sigma. Other reagents include 1% methylcellulose from Convergent, 90mM Phosphoric acid & 100mM Sodium hydroxide solutions in water.

Preparation of monoclonal antibodies, non-reduced conditions:

Samples were prepared for cIEF analysis on the iCE280 Convergent system by the addition of 190uL of water, 250uL of 1% methylcellulose, 20uL of a 10mg/mL sample, 20uL of ampholyte mix, 3-10/8-10.5 (1:1, vol/vol) & 2uL of pI markers, 7.05/9.5 (1:1, vol/vol).

Preparation of a 70kDa protein under reduced & denatured conditions:

A sample dilution buffer is prepared by mixing 2mLs of 10M Urea solution, 15mg solid Dithiothreitol (DTT), 120uL of 5-8 ampholyte, and 10uL of synthetic markers pI 5.30/6.60/7.00. The solution is mixed well and filtered using a millipore filter.

Reference STD & Sample preparation.

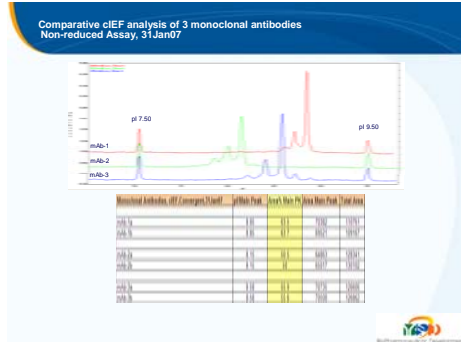
The 70kDa protein is first diluted to approximate concentration of 4mg/mL with HPLC grade water. Forty microliters of the above solution is further diluted with 480uL of the freshly prepared sample buffer. The sample is mixed and spun briefly for 1min.

Preparation of 10M Urea solution:

Weigh 30.0g +/- 0.3g Urea using an analytical balance. Transfer urea to a 50mL polypropylene tube. Add 25mLs of Milli-Q water and mix and sonicate until dissolved. Do not adjust the final volume (the actual concentration may be slightly above 10M)

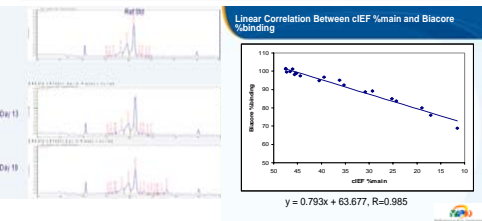
Applications

1. Comparative cIEF analysis of 3 monoclonal antibodies showed very reproducible results as to Area & Area% main peak, total Areas & isoelectric point (pI) of each molecule.



2. Investigation of a 13% decrease in binding observed by Biacore assay Result: By cIEF, a 12-13% decrease in Area% of the Main Peak (-52% to 40%) from day 13 to day 19 was observed with concomitant increase in low & high pI minor peaks.

cIEF Convergent	280	0	0	0	0	0	0			
Reference STD	50	mol/L	dil	10	mg/ml	8.12	28.5	48.9	82319	198382
DRCP73	Day13	8.13	24.1	52.4	102884	198371				
DRCP73	Day19	8.13	26.5	39.5	68444	173322				



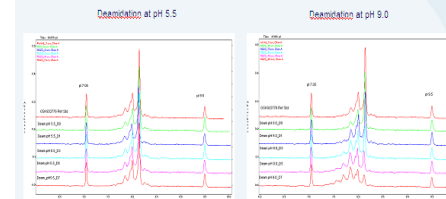
Applications cont'd:

3. cIEF assays to support Cell Line Selection

Investigated the presence of an early peak pI 8.2, in some cell lines, not observed in the reference STD. Decreased binding was also observed using the IGEN assay.

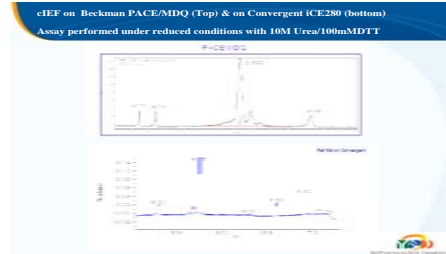
Cell Line Selection	280	0	0	0	0	0	0			
Ref Std	50	mol/L	dil	10	mg/ml	8.12	28.5	48.9	82319	198382
721	0.2	0.75	2%							
722	0.2	0.75	2%							
723	0.2	0.75	2%							
724	0.2	0.75	2%							
725	0.2	0.75	2%							
726	0.2	0.75	2%							
727	0.2	0.75	2%							
728	0.2	0.75	2%							
729	0.2	0.75	2%							
730	0.2	0.75	2%							
731	0.2	0.75	2%							
732	0.2	0.75	2%							
733	0.2	0.75	2%							
734	0.2	0.75	2%							
735	0.2	0.75	2%							
736	0.2	0.75	2%							
737	0.2	0.75	2%							
738	0.2	0.75	2%							
739	0.2	0.75	2%							
740	0.2	0.75	2%							
741	0.2	0.75	2%							
742	0.2	0.75	2%							
743	0.2	0.75	2%							
744	0.2	0.75	2%							
745	0.2	0.75	2%							
746	0.2	0.75	2%							
747	0.2	0.75	2%							
748	0.2	0.75	2%							
749	0.2	0.75	2%							
750	0.2	0.75	2%							
751	0.2	0.75	2%							
752	0.2	0.75	2%							
753	0.2	0.75	2%							
754	0.2	0.75	2%							
755	0.2	0.75	2%							
756	0.2	0.75	2%							
757	0.2	0.75	2%							
758	0.2	0.75	2%							
759	0.2	0.75	2%							
760	0.2	0.75	2%							
761	0.2	0.75	2%							
762	0.2	0.75	2%							
763	0.2	0.75	2%							
764	0.2	0.75	2%							
765	0.2	0.75	2%							
766	0.2	0.75	2%							
767	0.2	0.75	2%							
768	0.2	0.75	2%							
769	0.2	0.75	2%							
770	0.2	0.75	2%							
771	0.2	0.75	2%							
772	0.2	0.75	2%							
773	0.2	0.75	2%							
774	0.2	0.75	2%							
775	0.2	0.75	2%							
776	0.2	0.75	2%							
777	0.2	0.75	2%							
778	0.2	0.75	2%							
779	0.2	0.75	2%							
780	0.2	0.75	2%							
781	0.2	0.75	2%							
782	0.2	0.75	2%							
783	0.2	0.75	2%							
784	0.2	0.75	2%							
785	0.2	0.75	2%							
786	0.2	0.75	2%							
787	0.2	0.75	2%							
788	0.2	0.75	2%							
789	0.2	0.75	2%							
790	0.2	0.75	2%							
791	0.2	0.75	2%							
792	0.2	0.75	2%							
793	0.2	0.75	2%							
794	0.2	0.75	2%							
795	0.2	0.75	2%							
796	0.2	0.75	2%							
797	0.2	0.75	2%							
798	0.2	0.75	2%							
799	0.2	0.75	2%							
800	0.2	0.75	2%							

4. cIEF Analysis of Forced Deamidation of a monoclonal Antibody sample (pH 5.5, pH 7.0, pH 8.0 & pH 9.0 at 37°C) Shown below: Deamidation at pH 5.5 & pH 9.0

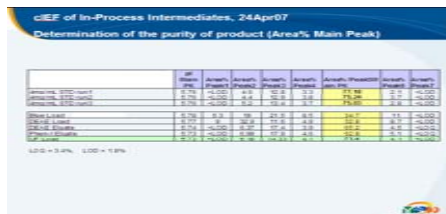


Results: No significant changes in protein separation patterns to day 7 at pH 5.5 incubation. The molecule showed almost complete degradation at pH 9.0.

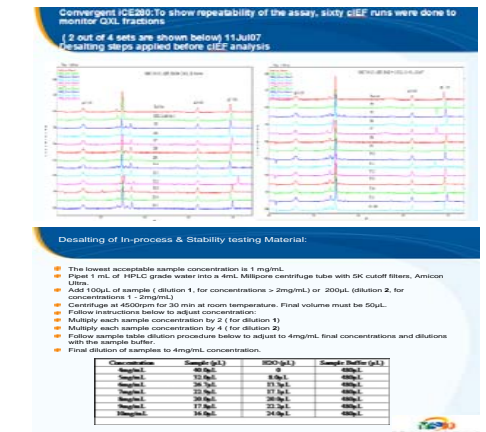
5. Compare charge variant separation profile of a reduced 70kDa protein: Result: Protein separation profile were comparable.



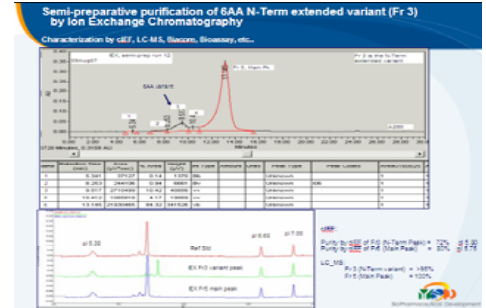
6. Determine purity of 70kDa product: Result: cIEF analysis of in-process material, from "Blue Load" material to final "UF Load" showed an increase in purity, the amount of the Area% Main Peak increased from ~35% to 71%.



7. Determine Repeatability of the cIEF assay



8. Characterization & Identification of a 6AA extended N-Term variant Method: by cIEF analysis, a variant with 6AA extra N-term sequence was detected in the new batch of reference std. An anion exchange method was developed to collect the Main Peak product (pI 5.75) and the N-Term variant (pI 5.82) and subsequently analyzed by cIEF & Mass Spec.



Conclusions:

The iCE280 cIEF system is a highly unique instrument for fast, reliable and an excellent method for separation of charged isoforms. The system provides the combined benefits of gel like separation with the automation and quantitation of column separation. It is very user friendly system, which is useful in analytical methods development, qualification & Technology transfer to QC and/or outsource contractors.

Acknowledgements

We are thankful for contributions provided by colleagues from various GSK departments: Bioseparation group, Immunoassay, Protein Characterization, Downstream Processing, Monoclonal & Microbial Cell Culture. We are most grateful for the efforts & support provided by experts from Convergent Bioscience.

References

- Wu, Jiaqi Method Development for cIEF Collaboration Project. Convergent Bioscience Ltd, Ontario Canada M8Z 2L8
- Huang, Tiemen Investigation of the Linearity and Stability of the pH Gradient Formed by Carrier Ampholytes with Whole Column Detection. Convergent Bioscience Ltd, Ontario Canada M8Z 2L8
- Zhang, M, Yim Ki Imaged cIEF As A Research Tool for Monoclonal Antibody Characterization and Development. Pharmaceutical Sciences, Faville Inc.