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# Separation and Characterization of a Monoclonal $IgG_2$ Antibody by Cation Exchange Chromatography

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Cation exchange chromatography (CEX) is a versatile method for separation of proteins based on exploiting differences in positive electrostatic charges. In CEX, proteins are bound to the negatively charged stationary phase (cation exchangers) and then eluted using a salt gradient. Typically, the liquid-phase pH in CEX is lower than the isoelectric points (pI) of the proteins.[1] CEX has been used to monitor various post-translational modifications such as glycosylation, deamidation, phosphorylation, truncation, oxidation, C-terminal and N-terminal clipping, and N-terminal cyclization.[2–10] Some of these variants may exhibit different bioactivity.[11] Therefore, it is important to characterize protein variants and monitor the stability of these variants throughout the process of drug discovery, development, and manufacture.

Characterization of complex proteins, such as antibodies, has traditionally been performed using slab gel-based techniques such as isoelectric focusing (IEF).[12] This technique is qualitative and time consuming. It also generates large quantities of chemical waste from the staining process. Turnaround time is usually one day or more. Recently, literature has documented newer techniques such as CEX, capillary zone electrophoresis (CZE), capillary isoelectric focusing (cIEF), and capillary electrochromatography for separation and quantitation of antibody charge variants resulting from post-translational modifications.[7,8,13–16,18] All these methods are automated and quantitative. Time for each run ranges from 20 to 45 minutes. Both high performance liquid chromatography (HPLC) CEX and capillary electrophoresis (CE) are highly recommended for high-throughput protein drug development, including antibodies.

Monoclonal antibodies contain two light chains and two heavy chains. At the C-terminus of the heavy chain, incompletely clipped lysine (K) or arginine (R) is usually present, resulting in three different variants. In the case of lysine (K) at the C-terminus, the major species observed include 0K (no lysine), 1K (one lysine), and 2K (two lysines). At the N-terminus, pyro-glutamic acid (pE), glutamic acid (E), and glutamine (Q) are possible species. The combinations of the C-terminal and N-terminal variants can theoretically produce ten different species without even considering any variations in sialylated or deamidated species. These complex mixtures of antibody can be separated by CEX.

A CEX assay was developed to separate and quantify charge variants due to C-terminal lysine and N-terminal variants in heavy chains of an $IgG_2$ antibody. This assay can separate sialylated species, deamidations, and structural isoforms. This assay has also been used to monitor antibody drug process development using two different cell lines. This article describes characterizations of the $IgG_2$ by CEX along with enzymatic digestion, peptide mapping, Edman N-terminal sequencing, and a cell based bioassay.

## Experimental Methods

A Waters HPLC 2690 separation module (Waters Corp., Milford, MA) was used for these experiments. UV absorbance was recorded at 280 nm for chromatograms. Peak area integration was performed through Waters' Millennium software. A ProPac WCX-10 analytical column (4 mm x 25 cm) and a ProPac WCX-10 guard column (4 mm x 5 cm) (Dionex, Sunnyvale, CA) were used for CEX separation. A pre-column filter and a frit are from Upchurch Scientific, Oak Harbor, WA. The column was held at room temperature during separations. Buffer A was 20 mM MES, pH 6.0 (MES, monohydrate, free acid; J.T.Baker, Phillipsburg, NJ). Buffer B was 20 mM MES with 70 mM NaCl, pH 6.0 (sodium chloride; J.T.Baker). Buffer C was 20 mM MES

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with 0.7 M NaCl, pH 6.0. Column storage buffer was buffer A with 0.05% azide (sodium azide; J.T.Baker). Milli-Q water (Millipore, Billerica, MA) or equivalent HPLC-grade water was used in all buffers. The flow rate was set at 0.7 ml/min. The buffer gradient went from 100% A to 100% B in 25 minutes and was kept at 100% B for 5 minutes. Then, the gradient immediately switched to 100% C for 5 minutes to clean the column with a high salt buffer. Lastly, 100% A was used to re-equilibrate the column for 10 minutes. Samples were diluted to 250 µg/ml in CEX buffer A. Injection load was 50 µg in 200 µl.

IEF conditions: Novex® IEF pH 3–10 gels were purchased from Novex, San Diego, CA. These IEF gels are 5% polyacrylamide, non-denaturing, and do not contain urea. Other reagents and procedures are described in the protocol.

Capillary isoelectric focusing (C-IEF) of the antibody was performed on a Beckman MDQ capillary electrophoresis instrument using a neutrally coated, 50-µm ID x 30-cm long capillary (eCAP; Beckman Coulter, Fullerton, CA). Samples were prepared at 300 µg/ml in a 0.2% ampholyte solution, pI 3.5–9.5 ampholines (Pharmacia, Peapack, NJ), with 0.2% hydroxypropyl methyl cellulose (HPMC, H-4649, Sigma, St. Louis, MO, 0.4% of HPMC was prepared in lab). Internal standards, pI 6.2 and pI 7.5 (Bio-Rad Laboratories, Hercules, CA), were included with each sample. All samples were microcentrifuged for 10 minutes to degas. Samples were held at 10° C and the capillary was kept at 20° C during the CE experiments. The CE analysis was performed at 25 kV with a 10-minute focusing period followed by a 25-minute mobilization step. The anolyte (prepared in lab) was 20 mM phosphoric acid, the catholyte (prepared in lab) was 40 mM NaOH.

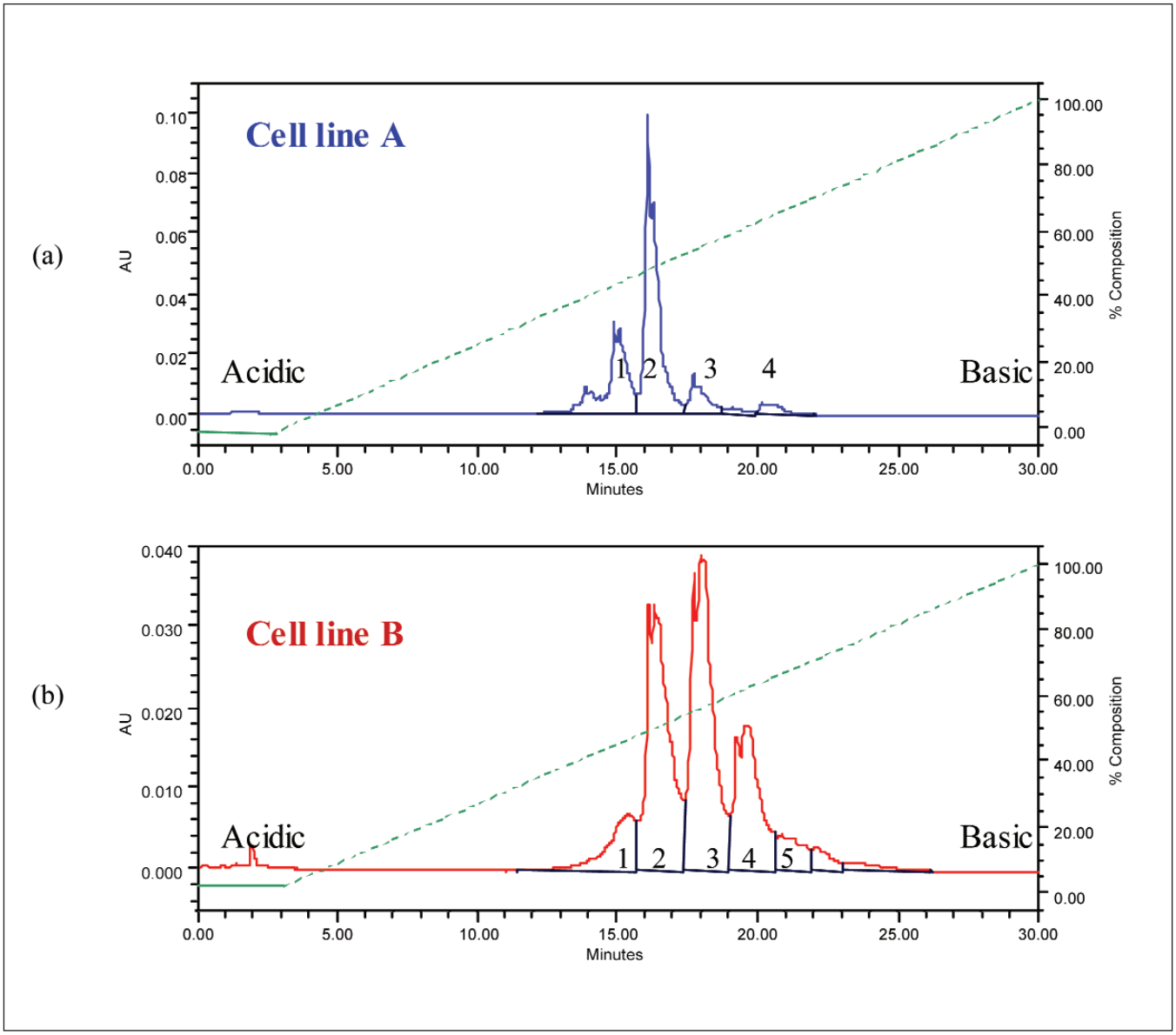



Figure 1. CEX chromatograms of an $IgG_2$ antibody from two different cell lines
(a) Cell line A; (b) Cell line B

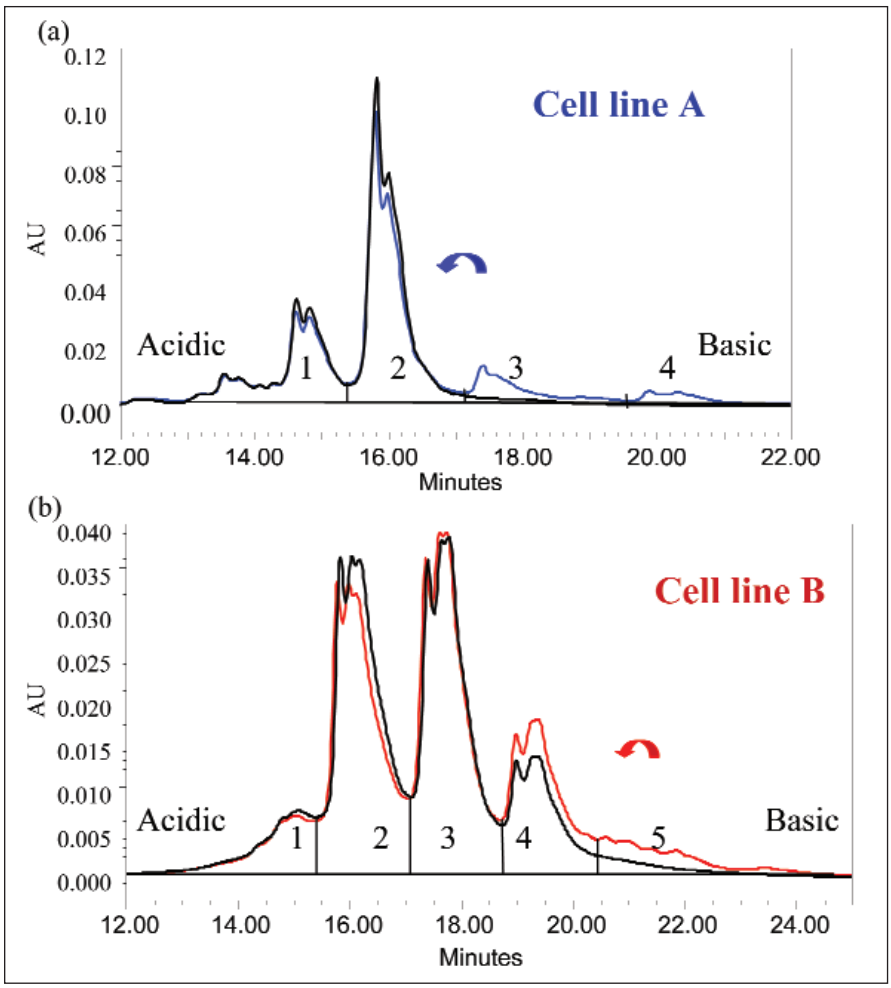



Figure 2. CEX chromatograms of an $IgG_2$ antibody from two different cell lines treated by carboxypeptidase B. (a) Cell line A ; (b) Cell line B. Black trace is from the treated sample.

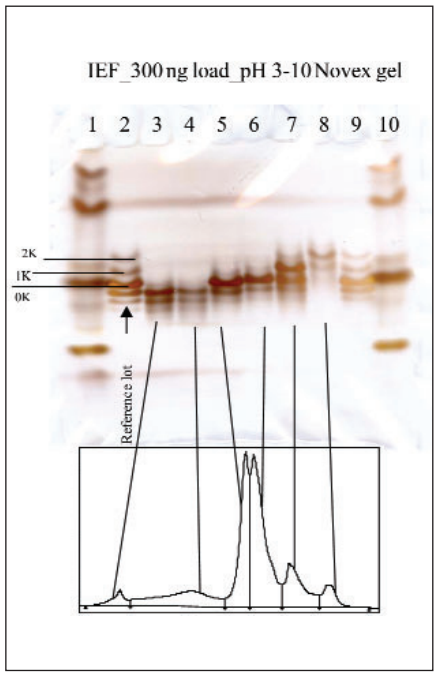



Figure 3. IEF gel of cell line A CEX fractions

Mobilization used a C-IEF Cathodic Mobilizer (Bio-Rad) in the cathode position. All buffers were sonicated in a water bath to degas prior to use. Elution was monitored at 280 nm.

Glyko sialidase and endoglycosidase-F2 (Endo F2) were purchased from ProZyme, San Leandro, CA; and carboxypeptidase B was purchased from Sigma-Aldrich, St. Louis, MO. All experiments involving sialidase, Endo F2, and carboxypeptidase were performed strictly according to standard protocols included with the enzymes.

Immobilized pepsin (ImmunoPure $F(ab')_2$) was purchased from Pierce Biotechnology, Rockford, IL. The reaction was carried out using conditions explained in the instruction provided with the preparation kit. Incubation time was increased to 20 hours at 37° C. $F(ab')_2$ was purified by an immobilized protein A column.

## Experimental Results and Discussion

### *CEX Chromatograms of One $IgG_2$ Antibody from Two Cell Lines*

CEX resolves the antibody into several peaks (Fig. 1). Peaks eluting earlier are acidic variants (peak 1) and peaks eluting later are basic variants (peaks 3–5). Clearly, the CEX profiles of materials produced from cell lines A (blue trace) and B (red trace) are significantly different. Interestingly, CEX resolves the major charged variants as doublets. The charge variants and doublets are indirectly characterized by enzymatic digestion using carboxypeptidase B, sialidase, Endo F2, and pepsin. Those enzymatic reactions were monitored by CEX and cIEF. Peptide mapping and N-terminal sequencing were used to confirm the results.

### *C-terminus*

To verify that the major peaks are due to variations of heavy chain C-ter-

**Table 1. Heavy chain blocked ratios from NTS of CEX fractions**

| | Recovery [pM] | | | |
|---|---|---|---|---|
| Sample | LC | HC | Ratio % LC/HC | ~% HC Blocked |
| Fraction 2 | 61.4 | 7.5 | 12.2 | 87.8 |
| Fraction 3 | 46.2 | 13.0 | 28.1 | 71.9 |
| Fraction 4 | 37.9 | 23.3 | 61.5 | 38.5 |
| Fraction 5 | 50.2 | 34.0 | 67.7 | 32.3 |

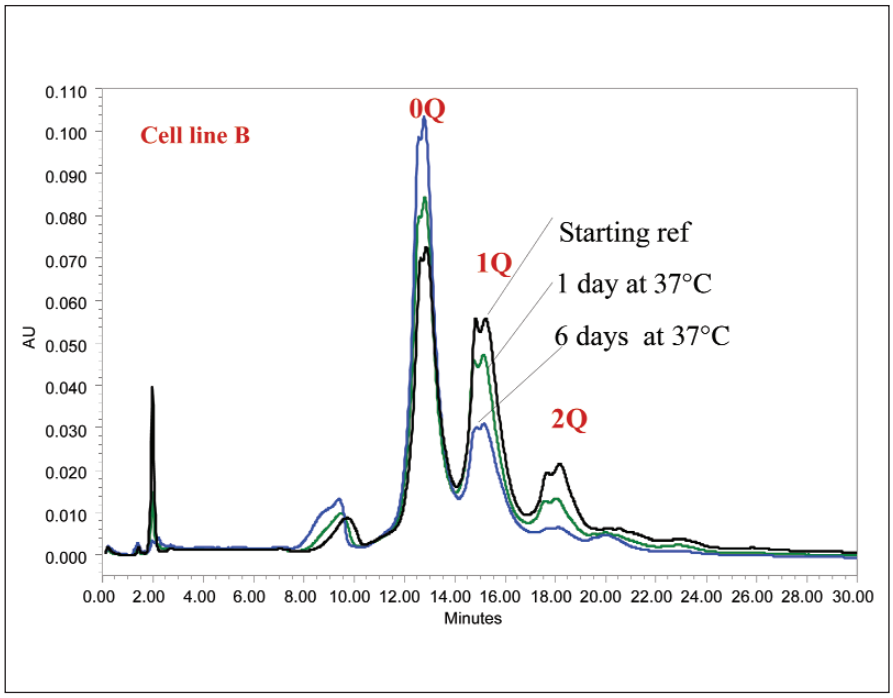



Figure 4. CEX of forced N-terminal cyclization progress

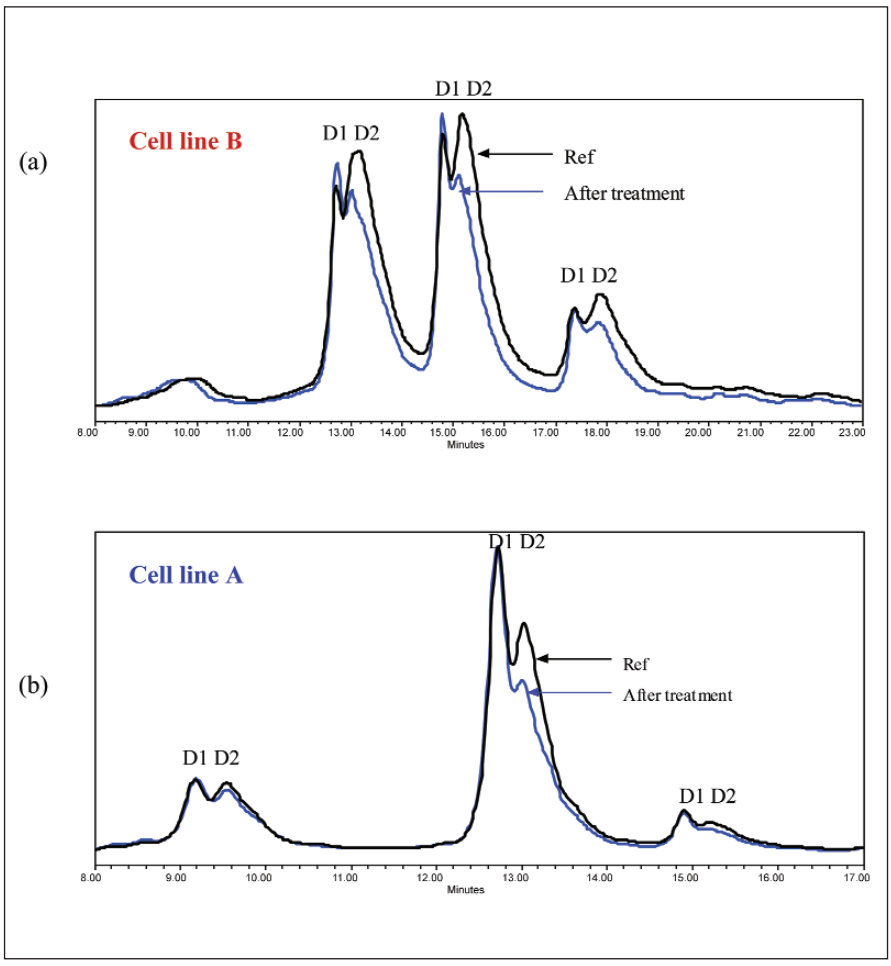



Figure 5. CEX of cell line A and B antibodies before redox (black trace) and after redox (blue trace) processes

minal lysine, the antibody was treated with carboxypeptidase B which specifically cleaves C-terminal lysine residues. If all charged variants are due to C-terminal lysine, then peaks 3–5 (Fig. 2) should collapse and co-elute with peak 2 (0K). Otherwise, peaks 3–5 are attributed to other positively charged variants.

Experimental data of cell line A antibody shows that peaks 3 and 4 collapse into peak 2, suggesting that peak 3 and peak 4 are due to lysine 1K and 2K, respectively (Fig. 2a). The doublets of both untreated and treated samples are unchanged. The origin of the doublets will be discussed later.

CEX fractions of the cell line A antibody were collected and analyzed by IEF gel electrophoresis. Figure 3 shows the image of IEF silver-stained gel for CEX fractions. Lanes 1 and 10 are IEF markers. Lanes 2 and 9 are from cell line A starting materials. Lanes 3–8 are from different CEX fractions. The corresponding semi-preparative CEX separation is shown in the bottom part of Figure 3. The cell line A starting material is clearly resolved into six major bands in the IEF gel (Lanes 2 and 9). The darkest band in the middle is likely due to 0 lysine, and the top band and the second band are due to 2 lysines and 1 lysine, respectively. This supports the assignment of 0K, 1K, and 2K for cell line A antibody. The doublets are not artificial but real because the fractions of those two doublets have slightly different migration times in the IEF gel. More importantly, when these fractions were reinjected back onto the CEX column, they exhibited different retention times.

Antibody produced by cell line B was also treated with carboxypeptidase B in the same set of experiments as cell line A antibody (Fig. 2b). Both CEX and IEF indicate that cell line B produced antibody contains little C-terminal lysine.

### *N-terminus*

CEX fractions were collected from the injection of 1 mg cell line B antibody. The fractions were concentrated and analyzed by N-terminal sequencing and peptide mapping. If the N-terminus is blocked, that would indicate cyclization of the N-terminus from Q to

pE. Edman N-terminal sequencing (NTS) of fractions 2 to 5 shows a heavy chain blocked ratio ranging from 87% to 32%, suggesting that a significant amount of heavy chain is not pyro-glutamic acid (pE) especially in peaks 3–5. Edman N-terminal sequencing and peptide mapping confirm the results of carboxypeptidase B digestion and CEX.[20] Data from NTS are included in Table 1.

In addition, N-terminal cyclization was monitored online by CEX (Fig. 4). These samples were kept at 37° C in the HPLC auto sampler. The HPLC program was set to inject the sample at a certain time and record the chromatograms. Figure 4 shows that peaks due to one glutamine species (1Q) and two glutamine species (2Q) decrease as the samples were kept for longer time. Eventually, those peaks can almost be fully reduced, indicating full cyclization is possible.

### *Structural Variants*

We observed doublets in major CEX peaks of the whole molecules and the $F(ab')_2$. We were not able to detect any differences in those doublets when analyzed by reduced peptide maps, a free thiol assay, BIAcore analysis, and bioassays. Therefore, redox experiments were applied to both cell line produced antibodies. The redox process allows mild reduction, conformational rotation, and oxidation. Reduction involves reducing mispaired disulfide bonds to free thiol (-SH) groups by addition of cysteine (CSH). Oxidation by addition of cystine (CSSC) converts the free thiols to correctly linked disulfide bonds to form more thermodynamically stable conformation.

After six days' incubation at 4° C in the presence of cysteine and cystine, the redox process was complete. Clearly, doublet D2 in Figure 5 was mostly converted back to doublet D1 with a remaining shoulder. Because doublet D2 was convertible to doublet D1 after the redox process, the structural variants represented by the doublets may be disulfide bond-related.

### *Acidic Variants*

Cell line A and B materials were treated with sialidase and Endo F2 to release sialic acids. The reaction mix-

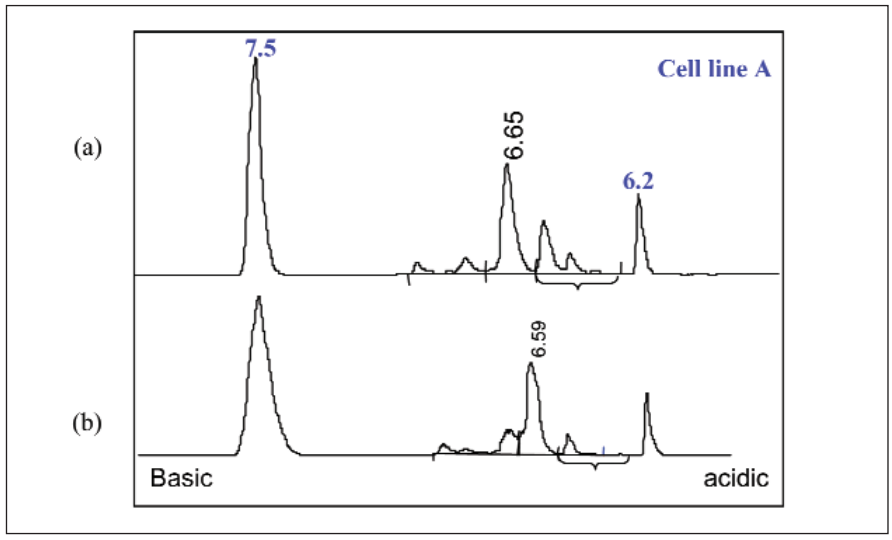



Figure 6. cIEF of cell line A. (a) Before treatment with Endo F2; (b) After treatment with Endo F2

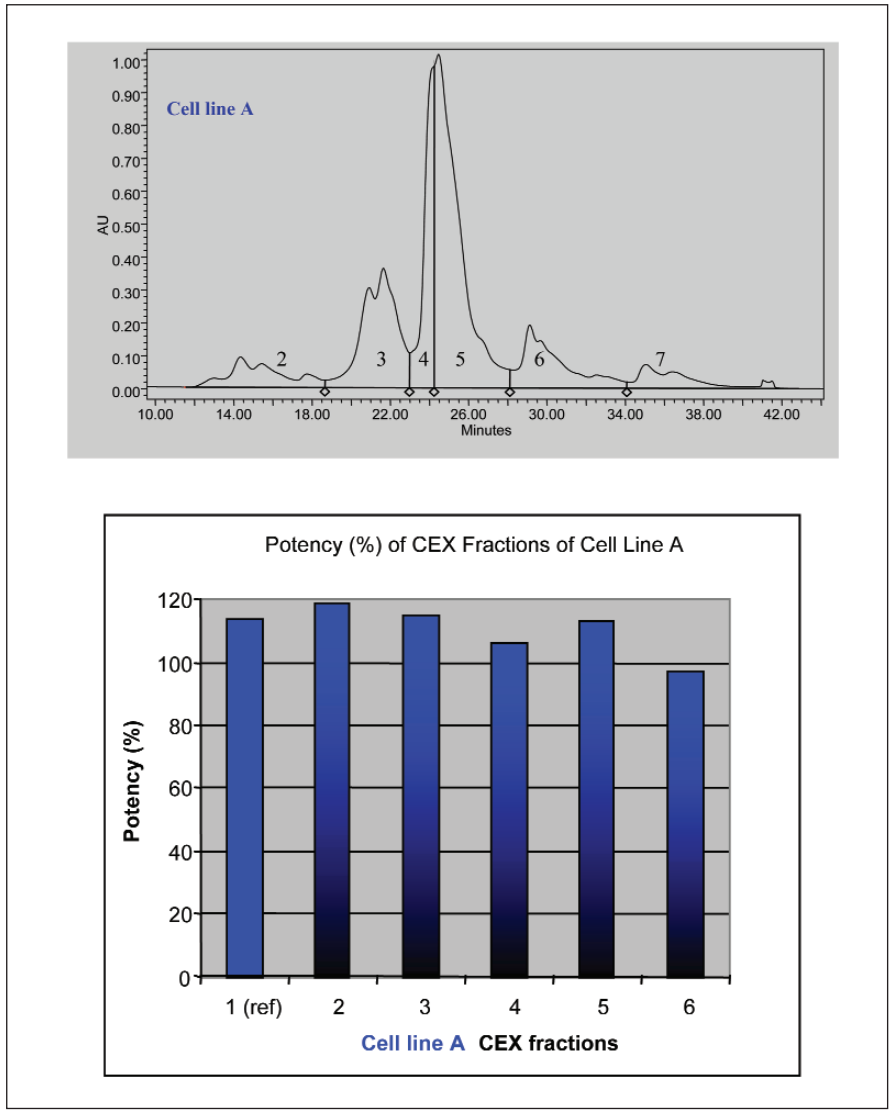
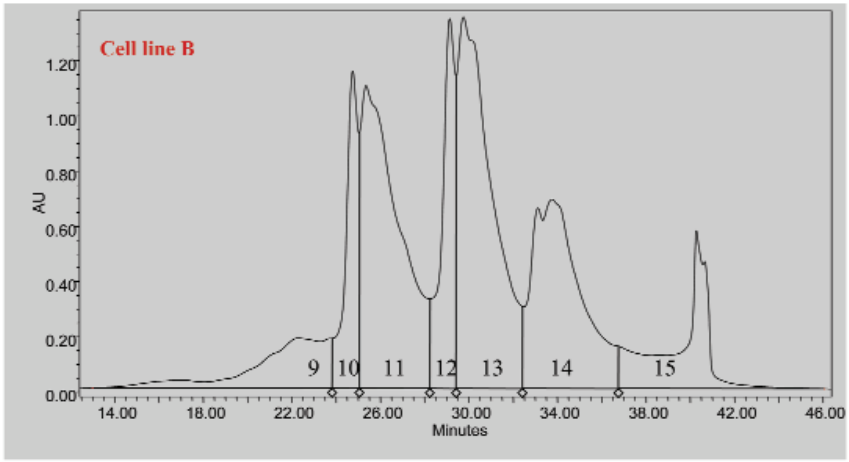



Figure 7. *In vitro* potency of cell line A CEX fractions

ture was separated by cIEF and CEX. After the sialidase treatment, about 30% of the total acidic variants are removed for cell line A antibody. The cell line A antibody then was treated with Endo F2, which cleaves within the N-acetyl-chitobiose core of high mannose and biantennary complex type asparagine linked glycans. Consequently, it removes all N-linked glycans without reducing the asparagine to aspartic acid. The reaction mixture was separated by cIEF (Fig. 6) and CEX (data not shown). In the figure, pIs of 7.5 and 6.2 are small molecule pI markers. After Endo F2 treatment, 80% of the total acidic variants are reduced for cell line A antibody, indicating that the majority of acidic variants are caused by sialic acid on the N-linked glycans. Overall, for cell line A antibody, acidic variants represent about 40% of total material. Approximately 80% of the acidic variants can be attributed to sialic acids. The remainder of the acidic variants may be attributed to deamidation or other unknown post-translational modifications. Few changes were observed for cell line B antibody when treated with sialidase or Endo F2, suggesting insignificant sialylation modification.

Immobilized pepsin was used to generate $F(ab')_2$. The reaction mixture was purified by a protein A column to obtain $F(ab')_2$. After cleaving the Fc from $IgG_2$ of cell line B, the amount of cell line B acidic variants do not change; whereas with cell line A, the acidic variants decrease dramatically, suggesting that most of the acidic variants of cell line A are located in the Fc domain, which is consistent with the variants being related to the N-linked sialylated glycans (data not shown).[20] In addition, the doublets are still preserved in the $F(ab')_2$, suggesting the structural variants are caused by $F(ab')_2$.



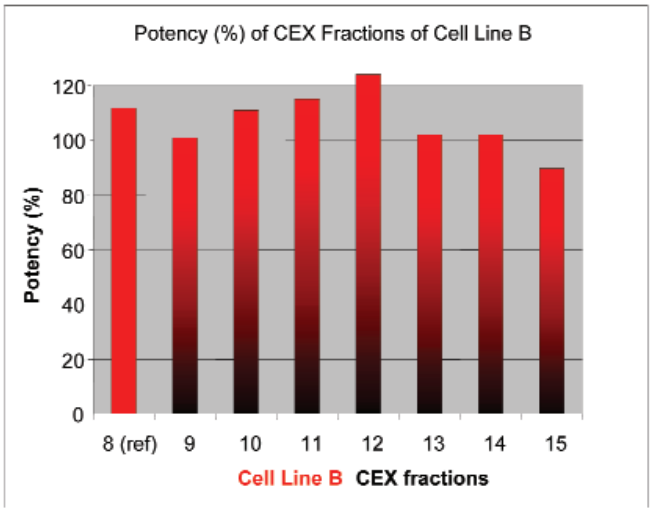




Figure 8. *In vitro* potency of cell line B CEX fractions

### *Potency*

Many different variants including N-terminus, C-terminus, and structural isoforms have been described and characterized. The most important aspect to determine is if the potency varies. Therefore, the fractionated CEX peaks were tested using an *in vitro* cell-based potency assay, a binding assay, and BIAcore. All assays show no differences among those variants. The potency data and semi-preparative CEX are shown in Figures 7 and 8. The potency of CEX fractions of cell line A samples 2–6 and B samples 9–15 is assumed to be the same as the controls 1 and 8. Too little fraction 7 was available for bioassay.

### Summary

CEX separates acidic, C-terminal lysine, and N-terminal glutamine/pyroglutamic acid variants of $IgG_2$ antibody. The acidic variants of cell line A represent about 40% of the total material. Approximately 80% of those acidic variants can be attributed to sialic acid in cell line A antibody, whereas little sialic acid was found in cell line B material. The remainder of the acidic variants may be attributed to deamidation or other unknown post-translational modifications. CEX also partially resolves the $IgG_2$ antibody into doublets. These doublets are associated with the $F(ab')_2$ domain. The doublets in the CEX are structural variants related to disulfide bonds. Overall, all the variants resolved by CEX of this $IgG_2$ (including acidic, C-terminal, N-terminal, and structural variants) exhibit comparable *in vitro* potency.

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