Charge Variant Analysis of USP Monoclonal Antibody Reference Standards Using icIEF

Monoclonal antibodies (mAbs) play an ever-increasing role in the pharmaceutical market. Biopharmaceutical companies must characterize quality attributes of their mAbs to ensure product safety, potency, and consistency. For mAb products, charge variants have been identified as critical quality attributes (CQAs) that must be assessed throughout development and the commercial product lifecycle to meet the regulatory requirements. The process and analytical control for charge variants can be challenging due to the heterogeneity from both post translational modifications (PTMs) such as glycosylation and C-terminal lysine clipping as well as chemical modifications such as oxidation and deamidation.

Several PTMs which occur during biosynthesis confer variation in charge, either by direct charge difference or by inducing conformational changes. These changes can impact quality, stability, and potency of a mAb. N-terminal modifications which can affect charge include cyclization of N-terminal glutamine (Gln) or glutamate (Glu) to form pyroglutamate (pyroGlu). C-terminal modifications include the removal of C-terminal lysine (Lys) and the amidation of proline (Pro). Cysteine related modifications can also affect charge, including the presence of reduced cysteine, alternative disulfide bond linkage, and formation of trisulfide bonds. Sialylated glycans may also contribute to charge variants.

Trimming of C-terminal lysine

The presence or absence of heavy-chain C-terminal lysine is an important metric for monitoring process consistency. After biosynthesis, mAb C-terminal lysine is enzymatically trimmed by endogenous enzymes, a process that occurs quickly in vivo (half-life ~1 hour) but is highly variable during recombinant production[1]. Despite the importance of C-terminal lysine profile for process understanding, it is rarely a CQA as no therapeutic significance has been attributed to its presence or absence.

Degradation during processing and storage can change mAb charge profiles. Charge profile analysis is commonly part of stability characterization studies and monitoring strategies. Major degradation pathways include deamidation of asparagine (Asn) to aspartate (Asp) and isoaspartate (IsoAsp). Isomerization of Asp may also produce succinimide residues as a deamidation intermediate. Glycation of lysine or the N-terminus of the light or heavy chain can also result in charge variants.[2,3]

Deamidation of asparagine

Deamidation of asparagine to aspartate or isoaspartate is a common degradation pathway that decreases mAb pI. Conditions of elevated temperature and high pH increase the frequency of deamidation events and can be encountered during IEX chromatography or pH neutralization following protein A elution under acidic conditions. Deamidation is a common CQA as it can impact conformational stability, binding affinity, and effector function.[4]

There are multiple analytical techniques like ion exchange chromatography (IEX), Capillary isoelectric focusing (cIEF) or imaged capillary isoelectric focusing (icIEF) used for charge variant analysis. This technote is focused on icIEF.

icIEF provides:

- Isoelectric point (pI) values and charge profile to support identity
- Quantitation for purity (quantitative or semi-quantitative)
- Relative percent of acidic group, basic group, the main peak, and individual species of interest

To support analytical development for mAbs, USP has developed three non-compendial monoclonal antibody Reference Standards (mAb 001, mAb 002, and mAb 003) with different physicochemical properties **(Table 1)**[5] and a variety of PTMs yielding unique charge profiles at a range of isoelectric points.

TABLE 01

General information for the three non-compendial USP mAb Reference Standards

	USP mAb 001, monoclonal IgG1	USP mAb 002, monoclonal IgG1	USP mAb 003, monoclonal IgG1
USP Catalog #	1445539	1445547	1445595
CAS #	174722-31-7	216974-75-3	912628-39-8
MW	~147,000 Da	~150,000 Da	~146,000 Da
Package size	200 µl solution (2 mg protein content)	200 µl solution (2 mg protein content)	200 µl solution (2 mg protein content)

See general information application note for more details[4]

All three mAbs are recombinant humanized IgG1 isotype expressed in Chinese hamster ovary (CHO) cell culture and produced by common industry upstream and downstream purification processes. These well-characterized mAb materials can be used in support of analytical methods for monitoring CQAs during the product life cycle, development of platform technologies, monitoring method performance (internal assay control), standardization across laboratories, training, and analytical method transfer.

Characterization of the USP mAb RSs included peptide mapping, which confirmed the presence of PTMs that impact charge **(Table 2)**. All three mAbs contain <0.05 nmol:nmol of sialic acid (Neu5Ac and Neu5Gc), making the contribution of sialic acid to the charge variant profiles negligible.

TABLE 02

Post-translational modification (PTM) analysis – Pyroglutamate and Lysine truncation

USP Reference Standard	PTM %		
	Light Chain	Heavy Chain	
	N-term Pyro Glu	N-term Pyro Glu	C-term Lys truncation
mAb 001	95.9	98.7	95.7
mAb 002	-	1.1	98.6
mAb 003	-	1.5	90.7

To support analysis of the charge heterogeneity of USP mAbs, we developed a method for icIEF using Bio-Techne's Maurice and iCE3 platforms **(Table 3)**.

The pI and charge profiles of the three mAbs were determined using both icIEF platforms in a multi-laboratory study. Intermediate precision between labs for main peak pI did not exceed +/- 0.2 pI units for all mAbs. Additionally, similar charge profiles were observed between collaborators. As shown in **Table 4** and **Figure 1**, mAb 002 and mAb 003 have a lower pI relative to mAb 001 and each mAb shows a unique profile of acidic and basic variants. The sample preparation and separation conditions were optimized for evaluation of pI and separation of charge variants in the USP mAbs. The availability of three mAb standards with different pI and charge profiles allows selection of the mAb that most closely reflects a developer's specific attributes of interest.

TABLE 03

icIEF Method for USP mAb RS Analysis

	icIEF
Sample Preparation	Intact mAb
Sample buffer	Pharmalyte 5-8 and Pharmalyte 8-10.5 (1:3)
pI standard	pI 6.14 and pI 10.17
Focus Period & Capillary voltage	Focus Period 1: 1 minute, 1500 V; Focus Period 2: 10 minutes, 3000 V
Sample Load Duration	55 seconds
Detector	Fluorescence and UV280 nm

FIGURE 01

Charge profile determined by Maurice icIEF. Electropherograms are representative of two instrument models, multiple preparations, and multiple injections from three independent laboratories.

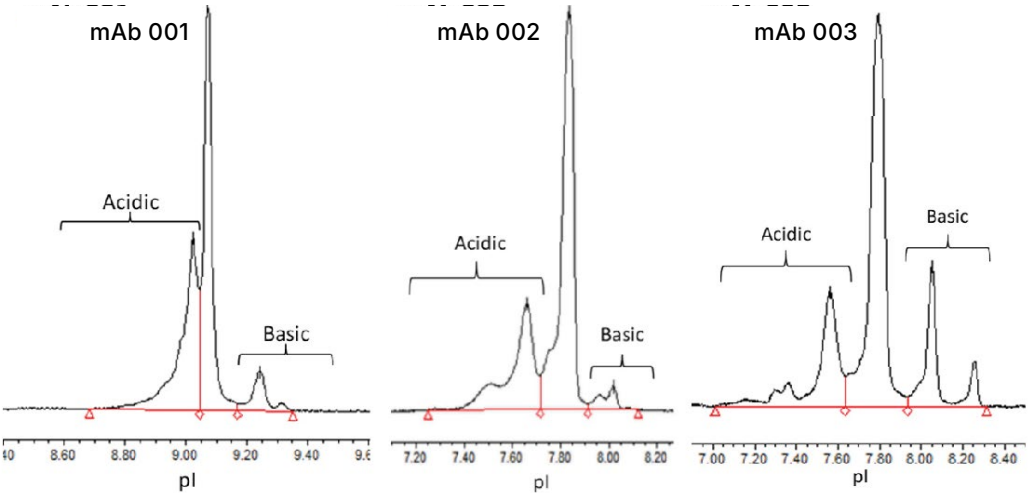

TABLE 04

Theoretical and Experimental pI values of main charge variants determined by icIEF

Reference Standard	Theoretical pI*	Experimental pI (icIEF)	Main (icIEF)	Acidic (icIEF)	Basic (icIEF)
mAb 001	8.7	9.2	54%	38%	8%
mAb 002	8.1	7.9	66%	29%	4%
mAb 003	8.1	7.9	62%	20%	18%

Note: Main peak pI and % species vary based on capillary condition, reagents, instrument, method, and integration parameters. Values are the average from three labs.
*Calculated using ProtParam (ExPASy) without glycosylation

Conclusions

The USP mAb Reference Standards provide users with options to find the best fit-for-purpose RS for their product.

USP mAb reference standards support method development and system suitability for charge variant characterization, release testing, and stability testing.

The range of pI and unique profiles of charge variants of the three mAb RS make them highly versatile and suitable for a broad number of uses and applications.

These mAb Reference Standards are another example of USP's continuous commitment to providing a foundation for high quality medicines.

Ordering Information

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