

A Novel *in vitro* Serum Stability Assay for Antibody Therapeutics incorporating Internal Standards

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PURPOSE

In vitro stability assessment plays a pivotal role in proactively identifying potential liabilities of antibody therapeutics prior to animal studies. The liquid chromatography-mass spectrometry (LC-MS)-based assays typically involve 3 steps: incubation of antibodies in biological matrices, affinity purification, and LC-MS analysis. To our best knowledge, there are no reported instances of routine *in vitro* stability screening methods involving internal standards which could be applied to antibody therapeutics across different biological modalities. Operational errors and uncompensated variations in sample evaporation, protein precipitation, sample recovery after affinity purification, and matrix effect during LC-MS analysis could lead to inaccurate stability estimation. By incorporating internal standards, a more accurate stability assessment could be implemented for routine screening.

OBJECTIVES

There are two goals we aim to achieve by establishing this *in vitro* serum stability workflow: 1) improving assay data quality by incorporating internal standards; 2) defining the assay acceptance criteria with existing *in vitro* stability data of 19 antibody therapeutics.

METHOD

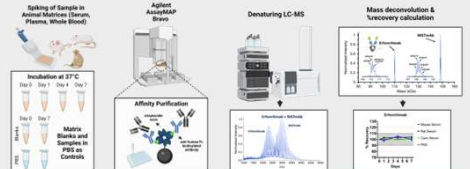
Two internal standards, NISTmAb, a recombinant humanized IgG1k, and Fc fragment of NISTmAb were established in our *in vitro* serum stability method. Fc fragment of NISTmAb was produced in house by IgD digestion of NISTmAb, followed by protein A purification. The stability of 19 monoclonal or bispecific antibodies, either in clinical use or clinical trials, were incubated with internal standards for 7 days in serums of preclinical species such as mouse, rat and cynomolgus monkey. All the *in vitro* samples were affinity purified using Bravo AssayMAP platform (Agilent Technologies, Santa Clara, CA) with goat anti-human IgG (anti-Fc). Triplicate injections of each purified sample were performed on a PLRP-S column (1000 Å, 2.1 × 50 mm, 5 µm) in 1290 Infinity II LC system coupled to 6545xt qTOF mass spectrometer (Agilent Technologies, Santa Clara, CA). Deconvolution of mass spectra and integration of mass peak area of deconvoluted masses were performed in Byos, version 4.5 (Protein Metrics, Cupertino, CA). The mass peak area ratio of antibody and internal standard was calculated with Equation 1.

$$\text{Mass Peak Area Ratio} = \frac{\text{Antibody Mass Peak Area}}{\text{Internal Standard Mass Peak Area}} \quad (1)$$

Percent (%) recoveries were calculated with Equation 2, by normalizing the mass peak area ratios of samples collected on Day 1, Day 4 and Day 7 to samples collected on Day 0.

$$\text{Percent Recovery} = \frac{\text{Mass Peak Area Ratio on Day X}}{\text{Mass Peak Area Ratio on Day 0}} \times 100\% \quad (2)$$

We adopted the general acceptance criteria for hybrid immunoaffinity-LC-MS/MS quantitative assay precision (within 20.0%) and accuracy (within ±20.0%) for initial data evaluation.



RESULTS

NISTmAb as Internal Standard

NISTmAb showed favorable stability in serums of mouse, rat and cynomolgus monkey across 7-day incubation time (Fig 1). The mouse serum stabilities of 9 bispecific antibodies were evaluated with NISTmAb as internal standard, as shown in Fig 2. The accuracies of %recoveries of these 9 molecules in this set of data ranged from -22.5% to 3.1% without internal standard, compared to -11.0% to 8.7% with internal standard. The precisions remained in the same range (0.3% to 4.3%) without internal standard. The data qualities of Tibuzumab, Tidutabam, INBRX-105, Zanidatamab, CTX-009, Tarlatamab, and Glofitamab were improved with internal standard. On the other hand, the accuracies of %recoveries of Erfonilimab and TNB-738 were well within ±10.0% without utilization of internal standard, representing cases where variations and operational errors occurred at a limited level or frequency during sample preparation and instrumental analysis. In these two cases, the data quality was not impacted by applying internal standard, again demonstrating that NISTmAb qualified as a good internal standard in this workflow.

NISTmAb was not an ideal internal standard for antibodies of similar sizes, which was illustrated by the case of Amivantamab. NISTmAb coeluted with Amivantamab during LC separation (Fig 3A), resulting in overlapping MS1 spectra (Fig 3B). The deconvoluted masses of summed MS1 spectra in Fig 3B was a combination of both NISTmAb and Amivantamab, shown in Fig 3C. It was difficult to accurately integrate the mass peak areas of either NISTmAb or Amivantamab.

Fc Fragment as Internal Standard

The Fc fragment was evaluated in the same fashion as NISTmAb and showed favorable stability in serums of mouse, rat and cynomolgus monkey across 7-day incubation time (Fig 4). The mouse serum stabilities of 6 monoclonal antibodies and 6 bispecific antibodies were evaluated with Fc fragment as internal standard, as shown in Fig 5. When internal standard was not utilized, the precisions were between 0.2% and 10.0% while the accuracies of %recoveries were all within ±10.0% except Palivizumab (-11.0% to -2.9%), indicating that variations and operational errors occurred at a limited level or frequency in this experiment. When internal standard was utilized in data analysis, the accuracies of %recoveries of Palivizumab, Amivantamab, and Erfonilimab were improved while the data qualities were not impacted for the rest of the molecules. The Fc fragment was demonstrated to be a suitable internal standard for antibody therapeutics as well as NISTmAb.

Selection of Internal Standard

The mouse serum stability profiles of Erfonilimab and Glofitamab generated with both NISTmAb and Fc fragment were compared, showing consistent trends (Fig 6). Fc fragment could be utilized as a universal internal standard, as the observed masses of Fc fragment do not overlap with any antibody therapeutics listed in Table 1. However, the in-house generation of Fc fragment is time consuming and costly. We haven't yet identified any commercial recombinant IgG1 Fc fragment with good stability in serums of preclinical species. NISTmAb remains the best option of internal standard for antibody therapeutics which are <140 kDa and >160 kDa.

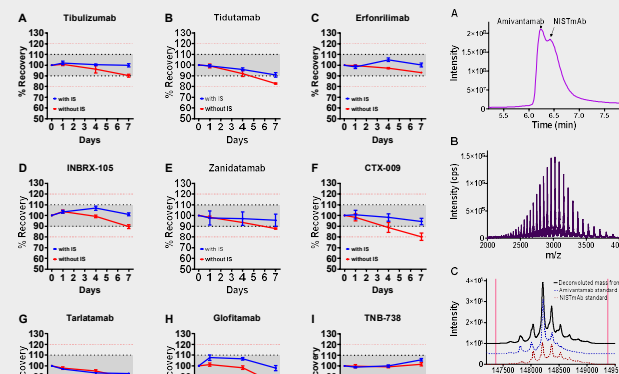


Figure 2. Result comparison: with and without NISTmAb as internal standard. A) Tibuzumab, B) Tidutabam, C) Erfonilimab, D) INBRX-105, E) Zanidatamab, F) CTX-009, G) Tarlatamab, H) Glofitamab, and I) TNB-738.

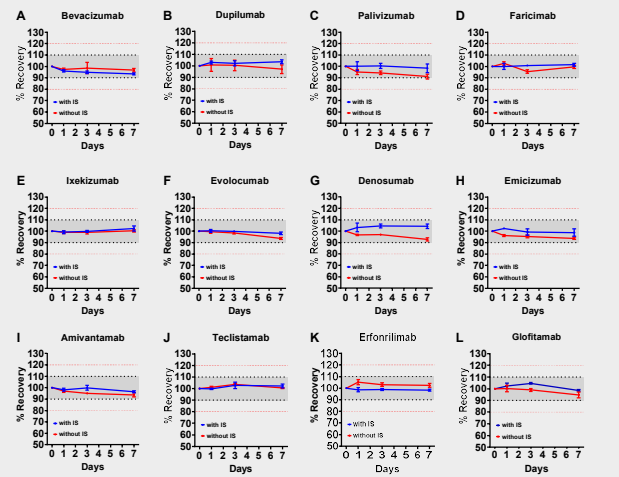


Figure 5. Result comparison: with and without Fc fragment as internal standard. A) Bevacizumab, B) Dupilumab, C) Palivizumab, D) Faricimab, E) Ixekizumab, F) Evolocumab, G) Denosumab, H) Emicizumab, I) Amivantamab, J) Teclistamab, K) Erfonilimab, L) Glofitamab

RESULTS (CONTINUED)

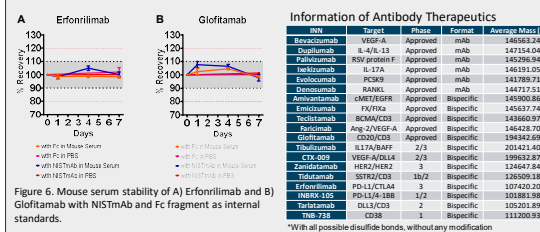


Figure 6. Mouse serum stability of A) Erfonilimab and B) Glofitamab with NISTmAb and Fc fragment as internal standards.

Acceptance criteria of antibody therapeutics with favorable serum stabilities

Prior to the incorporation of internal standards, broad acceptance criteria (accuracy at ±25.0%, precision at 20.0%) were used to recognize stable molecules during routine screening, to avoid deprioritizing antibody candidates of potentially good developability.

By incorporating internal standards, an acceptance criterion of 90.0% to 110.0% recovery within 7 days (accuracy at ±10.0%, precision at 10.0%) could filter out molecules with potential liabilities and offer confident stability outcomes. During routine screening, 85.0% to 115.0% recovery within 7 days (accuracy at ±15.0%, precision at 10.0%) is more inclusive and practical to recognize stable antibody therapeutics at the early stage of drug development.

CONCLUSION

With 19 stable antibodies, we demonstrated that the data quality of stability quantitation was improved by incorporating internal standards for *in vitro* serum stability assessment. This enabled confident stability assessment by monitoring the trend of %recovery of intact antibodies, in the absence of aggregation or biotransformation observed in LC-MS data.

This workflow has been routinely used in our laboratory to support early screening of large numbers of antibody therapeutics. In the case of a molecule with stability liabilities in serum, a continuous decreasing trend of %recovery of intact antibody correlated well with observed catabolites in LC-MS data or evidence of aggregation. However, due to the proprietary nature of these molecules, these data cannot be published at this moment.

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Disclaimers:
AbbVie sponsored this study; contributed to the design; participated in collection, analysis, and interpretation of data; and in writing, reviewing, and approval of the final version. Yihan Li and Hetal Sarvaiya are employees of AbbVie and own AbbVie stock. Rosendo Villafuerte-Vega is a former AbbVie summer intern and has no conflicts of interest.

