Middle-down Approach for Monitoring Monoclonal Antibody Variants and Deglycosylation
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ABSTRACT
The monoclonal antibody (mAb) therapeutics market is growing at a rapid rate owing to increasing demand for targeted treatments. Therapeutic mAbs are mostly produced from hybridoma cell lines. mAb production processes are complex and involve several critical steps, some of which are cell- and strain-specific. The final mAb products are heterogeneous, containing multiple charge variants and glycosylation forms. As a result, accurate and sensitive methods for mAb characterization and validation are essential. This study presents a Middle-down Approach for Monitoring Monoclonal Antibody Variants and Deglycosylation, a comprehensive approach for identifying mAb fragments containing charge, glycosylation, and oxidation modifications. The approach utilizes a combination of high-performance liquid chromatography, mass spectrometry, and computational methods to efficiently analyze mAb fragment data.

INTRODUCTION
With the recent growth of the biopharmaceutical industry, sensitive and fast methods are required to monitor mAb production processes. mAb production processes are complex and involve several critical steps, some of which are cell- and strain-specific. The final mAb products are heterogeneous, containing multiple charge variants and glycosylation forms. As a result, accurate and sensitive methods for mAb characterization and validation are essential.

RESULTS
mAb Charge Variant Analysis
A workflow to generate smaller mAb fragments such as light chain (LC) and fragment (Fc) is shown in Figure 2. Each of these fragments has a series of charge variants. LC and Fc are well separated by using different mobile phases. LC/MS analysis of mAb fragments such as light chain (LC) and fragment (Fc) is shown in Figure 3. Each of these fragments has a series of charge variants.

mAb Oxidation Variant Analysis
Prior to deglycosylation, the mAb sample was treated with 1 unit of IdeS protease to cleave the constant regions of the heavy chain. The deglycosylation reaction was performed using the deglycosylation cocktail at 40°C for 16 h. The deglycosylated LC was analyzed using LC/MS to detect oxidation variants. The deconvoluted result agrees well with the calculated MW based on the sequence of the modified residue.

mAb Deglycosylation Analysis
A workflow has been developed to monitor mAb deglycosylation. The workflow involves the following steps: (1) enzyme addition, (2) incubation at specific conditions, (3) sample treatment, (4) LC/MS analysis. The workflow can be used to monitor the deglycosylation process and ensure product safety and efficacy.

MATERIALS AND METHODS
Chemicals and reagents
Fabs and mAbs (positive controls) were purchased from Genentech (South San Francisco, CA). Fabs and mAbs (negative controls) were purchased from Rappaport Biologics (El Segundo, CA).

Sample preparation
mAbs were reduced using 100 mM dithiothreitol (DTT) for 1 h at 25°C. 

Sample preparation
mAbs were digested using 1 unit of IdeS protease for 1 h at 25°C. The digest was then incubated at 50°C for 120min.

Mass Spec: Q Exactive™ Plus
MS Detection: positive-ion mode
Operator settings: m/z range 1,000–4,000
Sheath gas 45 arb. units
Spray voltage 3.9 kV
AGC target 3
Microscans 10
S-lens level 55
In-source CID 40 eV

Table 1: MS conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
<td>0.5 mL/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>80 ºC</td>
</tr>
<tr>
<td>Mobile phase A</td>
<td>H2O/TFA (99.9 : 0.1 v/v)</td>
</tr>
<tr>
<td>Mobile phase B</td>
<td>MeCN/ H2O/TFA (90: 9.9 :0.1)</td>
</tr>
<tr>
<td>Gradient</td>
<td>0.5 mL/min</td>
</tr>
</tbody>
</table>

Mass spectra were acquired using Thermo Scientific™ Proteome Discoverer™ software version 1.4.4.0. The deconvoluted mass spectra were compared with the calculated MW of the modified residue. The deconvoluted result agrees well with the calculated MW based on the sequence of the modified residue.