Modular Antibody *de novo* Sequence Analysis using Multi-tier LC-MS/MS Data

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List of abbreviations:

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| --- | --- |
| CDR | Complementarity determining region |
| FR | Framework region |
| FR-CDR-FR | CDR with flanking FRs |
| LC | Liquid chromatography |
| MS | Mass spectrometry |
| BU | Bottom-up |
| MD | Middle-down |
| RT | Room temperature |
| Fab | Fragment (Antigen Binding) |
| Fc | Fragment (Crystallizable) |

# Abstract (249 / 250)

Antibodies form an important class of biomolecules that are produced by the immune system to defend us against infections. Their importance is underlined by their successful use as therapeutic agents, enabled by their production as recombinant monoclonal proteins (mAbs). Prior to development of an antibody lead, identification of the amino acid sequence needs to be achieved. Commonly B-cell sequencing is used to identify the DNA/RNA sequences that lead to the antibodies of interest, although only a small subset of the B cells produce antibodies that end up in circulation. More recently mass spectrometry-based (MS) methods have been used for sequencing, with the added benefit that this is a direct approach to extract the sequence of the protein in circulation, thereby potentially providing insights into post translational modifications. Both approaches have their implicit challenges, and the complete extraction of the amino acid sequence is still difficult to achieve. In MS-based approaches mostly shotgun proteomics has been applied, where the antibody is digested into peptides prior to identification. With such an approach, gaps in sequence coverage often arise, mostly in the complementarity determining regions (CDRs) of the antibody that are responsible for the recognition and binding of infectious agents. Here, we demonstrate that by combining shotgun proteomics with middle-down (MD) proteomics, where the protein or large fragments thereof are measured intact, these gaps can be filled and better information on the sequence can be extracted. We therefore developed and describe software solutions to iteratively integrate data from BU and MD proteomics.

# Introduction

Antibodies, or immunoglobulins, are one of the cornerstones of the human immune system and are abundantly present in various bodily fluids, such as serum, saliva, milk, the lumen of the gut, and cerebrospinal fluid (Schroeder & Cavacini, 2010). Because of their important role in combatting infectious diseases, immunoglobulins have been intensively studied and in the last decades have taken centre stage for the development of novel therapeutics (Kaplon & Reichert, 2021; Marks & Deane, 2020; Raybould et al., 2020). In the last decade, antibodies have become the best-selling drugs in the market, notably in 2018 eight of the top ten bestselling drugs were biologics.

New antibody leads for biotherapeutics can be extracted from various sources, such as immunized animals or recovered patients who carry pathogen neutralizing antibodies (Bornholdt et al., 2016; Corti et al., 2016; Valgardsdottir et al., 2021). The incredible potential for diversity of immunoglobulin molecules in the human body, with over 1015 theoretically possible sequences (Briney, Inderbitzin, Joyce, & Burton, 2019; Schroeder Jr., 2006), indicates that each antigen exposure may lead to a unique, personalized (polyclonal) antibody response. One way to chart the antibody repertoire is to sequence the B-cell receptors of all B cells that can produce antibodies. It is however thought that only a marginal fraction of all these B cells indeed produce immunoglobulin proteins that end up in circulation, making this a inefficient undertaking. Alternatively and more ideal, investigation and sequencing of antibodies occurs directly at the protein level (Hom, Tomar, & Tipton, 2022).

Mass spectrometry (MS) has become *the* method of choice for analysing protein mixtures (Aebersold & Mann, 2016; Altelaar, Munoz, & Heck, 2013), but sequencing polyclonal antibody mixtures still poses one of the major remaining challenges (Peng, Pronker, & Snijder, 2021; Sen et al., 2017; Srzentić et al., 2020). Most protein analyses by MS are performed by peptide-centric proteomics, also called shotgun- or bottom-up- (BU) proteomics, where the presence and relative abundance of proteins is inferred from peptides obtained by digesting the proteins with proteases, prior to sequencing. For the identification, this approach makes use of a protein sequence database to generate theoretical peptides from which the expected precursor mass and fragmentation spectrum is generated (Aebersold & Mann, 2003). A sequence database is however not available for the full repertoire of antibodies, as their sequences are the result of the recombination and mutation of several genes encoded for by many different alleles in each person. An option to sequence antibodies by shotgun proteomics is by using *de novo* sequence analysis, where peptide sequences are directly determined from the fragmentation spectra. The resulting short peptide *reads*, typically 5-25 amino acid residues in length, are assembled into longer *contigs* or even full-length *protein chain* sequences (Guthals, Clauser, & Bandeira, 2012; Sen et al., 2017; Tran et al., 2016). A factor that makes read assembly for antibodies particularly difficult is that the sequence of both the light- and heavy chain of an antibody are made up of alternatingly conserved and hypervariable sequence domains (Alberts et al., 2002; Charles A Janeway, Travers, Walport, & Shlomchik, 2001). Fortunately, the quality of software platforms for *de novo* sequence analysis of antibodies by MS is steadily increasing (de Graaf, Hoek, Tamara, & Heck, 2022). Virtually all published platforms make use of homologous sequence templates (Castellana, Pham, Arnott, Lill, & Bafna, 2010; Schulte, Peng, & Snijder, 2022; Sen et al., 2017; Tran et al., 2016), obtained by comparing experimental data to an immunogenetic database such as the IMGT (M.-P. Lefranc & Lefranc, 2020; M. P. Lefranc, 2003). The commercially available antibody sequencing platform Supernovo for example takes BU data as an input and returns a full-length sequence, along with the determined germline template sequences (Sen et al., 2017). Through recent development in software and mass spectrometry results of these approaches may now lead to correct sequencing, albeit only for monoclonal antibody samples (Peng et al., 2021). However, established software solutions in the field, including Supernovo, cannot yet sequence antibodies in polyclonal mixtures with equal success.

Recent advances in instrumentation, separation, sample preparation and computational power have facilitated protein-centric proteomics (also called top-down proteomics). This enables the simultaneous analysis of an entire protein chain, removing the need for protein inference (Toby, Fornelli, & Kelleher, 2016). This approach is very enticing as it side-steps the need for assembling peptide sequences into a full protein sequence. While the field has not yet matured to yield spectra that can routinely be used for confident *de novo* sequencing without additional data, the continuous advances made indicate that the future of antibody sequence analysis will surely include using these techniques as a complementary source of information to the more established peptide-centric (BU) analyses. One particularly striking example of this is the use of middle-down (MD) proteomics for antibody sequence analysis, which improves sequence coverage and reduces complexity of the spectra by cleaving the constant region of the heavy chain with high specificity (Johansson, Shannon, & Björck, 2008). Reports of sequencing components of polyclonal mixtures are currently released as proof-of-concept studies (Bondt, Hoek, et al., 2021; Dupré et al., 2021; Schulte et al., 2022), where most of the studies make use of some form of intact protein (fragment) analysis, pointing towards integrative workflows combining multiple MS approaches as the way forward.

Recently a tool to sequence polyclonal mixtures using only BU *de novo* peptides was reported. The tool, named Stitch, yields exciting results by resequencing an abundant clone from serum (Schulte et al., 2022). Here we describe an integrated approach that builds upon Stitch by integrating MD-MS data, with the aim of improved antibody sequencing. This workflow sequences a target chain, selected from deconvoluted MS1 spectra of reduced antibody chains, in a modular, three stage process based on germline domains (as defined in the IMGT residue numbering scheme (M. P. Lefranc, 1997)). Each stage deals with increasingly large sequence segments, first sequencing the framework regions (FRs), then CDRs with flanking FRs (FR-CDR-FRs), and ultimately full chain sequences (Figure 1a). To demonstrate the performance of this approach, we analysed three samples of various scales of complexity, namely: a pure therapeutic antibody, namely Trastuzumab, both in a monoclonal sample and in a mixture of three monoclonal antibodies, as well as a single abundant IgA1 clone endogenously present in the serum repertoire of a sepsis patient. We used these samples to test the effectiveness of the workflow, by reconstructing the known sequence of the Trastuzumab heavy chain to a high degree in the monoclonal sample as well as in the more complex mixture of three monoclonal antibodies. We next applied the approach to sequence an abundant IgA1 heavy chain in a highly diverse polyclonal sample of IgA1 clones present in the serum of a sepsis patient. We show how integration of MD-MS data can be used to resolve ambiguity in *de novo* sequence predictions, particularly in hypervariable regions, through determining the mass of the CDR and using this mass to filter candidate CDR sequences and confirm their pairing to the fragmented precursor chain. We hypothesize that such improvements will be particularly beneficial when analysing polyclonal mixtures of increasing complexity or when lower sample amounts are available. The algorithms supporting the analyses were programmed in the C# programming language and are freely available on GitHub.

# Results

Antibody sequencing by any source of information poses a tough challenge due to the hypervariable yet homologous nature of the vast amount of sequences. For example, reference databases are of little to no benefit when sequencing the hypervariable CDRs, which in turn makes assigning bottom-up reads to these regions extremely difficult. Using MD proteomics provides not yet a realistic alternative, as the fragment coverage in MD-MS, although superior to that of top-down (TD) MS, is still too limited for stand-alone *de novo* sequencing, although exciting progress is made for sequencing reduced light chains (Dupré et al., 2021; Melani et al., 2019). Here, our hypothesis was that by combining BU- with MD-MS data, and reference sequences from immunological databases, these sources of information can complement each other and be used to fill gaps not covered by the individual approaches. Therefore, we make use of MD-MS fragmentation spectra combined with the relatively conserved nature of the FRs to determine the molecular mass of the CDRs. This is subsequently used as a filter to substantially reduce the number of candidate CDR sequences while simultaneously confirming their pairing to the fragmented precursor target chain.

The workflow consists of three stages: we first consider only FR sequences, then the FR-CDR-FR sequences, and finally the full-length sequences. Each stage first generates a candidate pool of sequence-solutions by considering ambiguities left by the previous stage, then evaluates these candidates using the integrated evidence streams, and finally resolves the ambiguities by discarding candidates that do not have supporting evidence (Figure 1a). By starting with the FRs, which are relatively well-conserved sequence segments, and resolving ambiguities at this scale before moving to longer, more variable segments by joining adjacent FR candidates into FR-CDR-FR contigs, the size of the search space at each stage remains at manageable sizes (Figure 1b).

## Target mass determination and sample characterization by using MD-MS

To characterize the complexity of the samples and determine the precursor masses of the target chains, we collected middle-down LC-MS/MS data for all samples. Our middle-down approach was performed according to previously published protocols (see **Immunoglobulin capture and Fab generation**) (Bondt, Dingess, Hoek, van Rijswijck, & Heck, 2021; Bondt, Hoek, et al., 2021). These protocols yield Fab fragments by specifically cleaving the Fc portion of the heavy chain. The resulting fab fragments were then reduced before LC-MS/MS analysis, to yield separated LC and Fd chains. We then deconvoluted the MS1 spectra to assess the number of unique LC and Fd masses in each sample (Figure 2)

For the monoclonal sample, as expected, 2 highly abundant peaks were observed (originating from the separated LC and Fd chains), accounting for over half of the total deconvoluted intensity. When adjacent peaks in both mass and retention time (±50 Da and ±1 minute) are considered, this increases to over 90% with the remaining masses consisting of *background* peaks of less than 5% relative abundance (Figure 2a). For the mixture of 3 mAbs, likewise and as expected, six abundant peaks were observed. The abundance of the target chains (±50 Da and ±1 minute) made up ~33% of the deconvoluted intensity. The other clones make up a total of 50% of deconvoluted intensity and ~20% is background (Figure 2b). Lastly, for the polyclonal sample, the target clone (±50 Da and ±1 minute) made up less than 20% of deconvoluted intensity (Figure 2c). The data in Figure 2 highlight challenges in deconvoluting middle-down spectra, as we observe that the used deconvolution software does lead to accurate masses, as peaks are incorrectly grouped and averaged in the more complex samples. To obtain the most exact masses, we averaged the MS1 spectra recorded over the elution window of each target chain (Figure 2; highlighted in red) before deconvolution. This improved the mass assignments to within 30 ppm accuracy for the Trastuzumab Fd in the monoclonal and mix sample and yielded a target precursor mass of 24811.17 Da for the most abundant clone in the polyclonal sample extracted from serum. Similarly, the MS2 fragmentation spectra were averaged over the elution windows of the target chains and deconvoluted. This yielded 919, 265 and 469 deconvoluted fragment ion peaks for the monoclonal, mix and polyclonal sample respectively (Table S1).

## **Using multi-enzyme shotgun proteomics data for *de novo* sequencing**

As part of the analysis each sample was also measured by BU-MS, by digesting each sample with 4 proteases in parallel and collecting peptide-centric LC-MS/MS data. The resulting spectra were submitted for *de novo* peptide identification using PEAKS (Ma et al., 2003), yielding a total (*i.e.*, cumulatively from all protease treatments) of 14000, 27421 and 35003 *de novo* peptide reads for the monoclonal sample, the mixture of three, and the polyclonal sample, respectively (Table S1). To illustrate the growing challenges of sequencing through shotgun proteomics in more complex samples, we reconstructed the known sequence of the Trastuzumab heavy chain from the *recombinant* *benchmark samples* (*i.e.*, the monoclonal and mixture of three sample) using BU-MS data alone. To this end, the peptide reads for these samples were submitted to the *de novo* peptide assembly tool Stitch (Schulte et al., 2022). The resulting output for the monoclonalsample was nearly perfect (Figure 3a). However, the consensus sequence as obtained for the sample from the mixture of 3 mAbs contained 4 erroneous residue predictions in the FR2, and 6 in the CDR1 and CDR2 (Figure 3c). These errors were the result of low peptide coverage, caused by assigning reads to the wrong templates. This caused splitting of reads that belonged to the same chain. Furthermore, the unassisted germline recombination by Stitch failed to select the correct V-region for recombination, as it was not the highest scoring V-region in the mix sample. This standard de novo sequencing of a recombinant mAb, becomes already difficult when two other mAbs of equal abundance are spiked into the sample.

To tackle these issues, we ran Stitch again with refined templates (*i.e.* the consensus sequence as output by the initial Stitch run, or *template selection* run, rather than the germline sequence) and a lower score cut-off for the input reads (50 instead of 85). To ensure recombination of the correct V-region, we manually defined which V-region templates should be recombined by Stitch by providing refined templates equal to the number of abundant clones present in the MD data (1 and 3 for the monoclonal and mix sample respectively; Figure 2a and b). For the monoclonal sample we selected the best scoring V-region, IGHV3-66, as a refined template. For the sample of 3 mAbs we selected 3 V-region templates: the highest unique score (IGHV4-39), the highest score (IGHV4-30-4), and the highest score in a different family (IGHV3-66). This additional Stitch run, or *definitive* run, gave a major improvement for analysis of Trastuzumab in the 3 mAb sample, as it improved the depth of coverage 2 to 28-fold and raised depth of coverage above the dynamic cut-off (the depth of coverage at Cys104, Figure 3) for 13 out of 21 positions (Figure 3d). Pleasingly, these adjusted settings had no detrimental effects on the performance for the monoclonal sample (Figure 3b), although some ambiguity remained in the predicted sequence for Trastuzumab in the 3 mAb sample.

## **Integrating multiple evidence streams**

### **Performance on recombinant samples**

#### Framework region sequencing

Using the residue frequency tables (Figure S1) from both Stitch runs, as well as a residue frequency table generated from the IMGT database, FR candidate sequences were generated by converting ambiguous residues into sequence candidates (Figure S1). This yielded between 1 and 756 candidates per target FR (Table S2) and included the known correct candidate for all recombinant benchmark samples. These candidates were evaluated against experimental BU- and MD-MS evidence and ranked by a combination of the resulting scores. For BU-MS scoring, a score was used that represents the depth of coverage of exact sequence matches longer than 6 residues, weighted by match length (termed Shotgun-score; Table S3). For MD-MS scoring, a score was used that represents the overlap between theoretical fragments of the sequence and peaks in the MD fragmentation spectrum (MD-score; Table S3). The MD-score is obtained using a *sliding window* scoring algorithm, which slides theoretical fragments generated from a given (sub)sequence over the spectrum to find the best scoring position, and thus outputs the optimal prefix- and suffix- mass of a given contig (Figure S2). Candidates missing highly conserved residues (Cys23, Cys104) as well as terminal segment (*i.e.*, FR1 and FR4) candidates with illogical prefix- or suffix- masses were removed in a first pass filtering step. This reduced the candidate lists up to 10-fold, to a maximum of 90 candidates (Figure S3).

We further filtered the candidate pools to a maximum of 40 candidates (Table S2) without eliminating any correct candidates by manual inspection of the scores. For the monoclonal sample, the correct FR1 candidate was ranked #1 with a large discrepancy between scores (Figure S3). As FR2, FR3, and FR4 only had one candidate each, no selection was needed. However, it was encouraging to see that the sliding window algorithm was able to correctly determine the prefix masses for these contigs with a mass error that did not exceed 18 ppm.

The candidate pools for the mixture of 3 mAbs were reduced from 240, 756, 5 and 4 candidates to 40, 7, 1 and 2 candidates for al FRs respectively (Table S4). For FR1, we rejected 200 candidates in the first pass, leaving 40 candidates. No further filtering was possible, as fragment and read coverages were too low for confident filtering (maximum of 2 fragments and no read coverage past Cys23). The FR2 candidates had many overlapping scores (Figure S3) due to low read coverage of the N-terminal ambiguous residues (Figure 3c and d) and a near total overlap of theoretical fragments for these candidates. We rejected the lower MD-scores (106 vs 121), which represented the same fragments but without a fragment match on the second residue. This reduced the number of candidates from 756 to 90. Subsequent filtering using the Shotgun-score, rejecting all but the best Shotgun-score (9.4k), left only 7 candidates, representing a single remaining ambiguous N-terminal residue. For FR3, only 1 out of 5 candidates had the highly conserved Cys104, leading us to reject all other candidates. For FR4, we rejected all candidates not starting with the conserved Trp118 but considered the difference in Shotgun-score for the remaining 2 candidates too small to reject either.

#### Complementarity determining region sequencing

To determine the sequence of the CDRs, we extended the selected FR candidates into FR-CDR-FR candidates. All adjacent FR candidates were paired to obtain all possible neighbouring pairs. We then calculated the mass gap between each of these FR pair (which is equal to the theoretical molecular weight of the CDR sequence) using the prefix- and suffix- mass of each FR candidate. Each FR pair was converted into a set of FR-CDR-FR candidates by connecting the FRs with candidate CDR sequences. These candidate CDR sequences were generated by first connecting peptide reads that extend from the FRs into the CDR, then discarding the candidates that do not match the calculated molecular weight of the CDR at 5 Da tolerance (Figure S4). The resulting FR-CDR-FR candidates were scored and ranked using the MD- and Shotgun- score (Table S3).

The top 10 FR-CDR-FR candidates for each FR pair were manually evaluated based on the scores to select the most likely FR-CDR-FR candidates. For both recombinant benchmark samples, these candidates contained the correct sequence for CDR1, CDR2 and CDR3. For the monoclonal sample, 10 FR-CDR-FR candidates were generated per CDR (Table S2). The correct candidate for each CDR could easily be selected using the Spectrum and Shotgun-score (Figure S2). The selected candidates all had the top MD-score (255, 508 and 1561 for the CDR1, CDR2 and CDR3 respectively) and the best (CDR1 and CDR2) or second best (CDR3) Shotgun-score (137k, 56k and 122k respectively).

For the mixture of 3 mAb sample 1106, 49 and 20 FR-CDR-FR candidates were generated for the CDR1, CDR2 and CDR3, respectively (Table S2). Despite the much larger starting pools, the correct CDR1- and CDR2- candidates could be selected unambiguously during manual inspection as they had the second best and best MD-scores (143 and 257 for CDR1 and CDR2 respectively) and the top Shotgun-score (30k and 40k respectively) with enough score difference to reject the other candidates (Figure S3). The selected FR-CDR-FR candidates for CDR1 also caused rejection of the remaining incorrect FR1 and FR2 candidates, which left only 7 FR-CDR-FR candidates for CDR2 as the rest did not contain the right FR2.

Scoring for the CDR3 was more ambiguous. Fragment coverage was insufficient to make a distinction between the FR-CDR-FR candidates, as MD-scores ranged only from 280 to 282. The Shotgun-scores were distributed in two clusters based on which FR4 candidate was included (Figure S3). The correct FR4 (starting with WGQGT) scored ~221k while the incorrect FR4 (starting with WGQGS) scored higher (~244k). However, we noted that the candidates with the wrong FR4 lacked connecting reads between the FR4 and CDR3. The candidates with the correct FR4 sequence had fewer but longer and more overlapping reads which connected the CDR3 and FR4 better (average read length of ~25 vs average read length of ~12). We rejected the higher Shotgun-scores on this basis.

The candidate pool with the correct FR4 included 2 incorrect FR-CDR-FR candidates, SR***WNDG***FYAMDY and SR***DNWG***FYAMDY, that were nearly identical to the correct candidate, SR***WGGDG***FYAMDY. We selected these 3 candidates based on the presence of longer and more overlapping reads in the CDR3 than the other 7, same as above. However, we could not discriminate between the 3 isobaric candidates at this point, leaving 3 candidates for the CDR3.

#### Full chain sequencing

We next expanded the scope to the entire target chain to verify the selected FR-CDR-FR candidates. To achieve this, we recombined all remaining FR1 to FR4 candidates and transformed these FR-sets into full length chain candidates by joining the FRs with CDR candidates in the same manner as before (see **Complementarity determining region sequencing**; Figure S4). The resulting chain candidates that deviated more than 5 Da from the precursor mass in the MD-MS data were discarded. To ensure that the selected FR-CDR-FR candidates indeed represented the best predictions, all resulting chain candidates were scored and ranked using the MD- and Shotgun- score (Figure S3).

This recombination yielded 930 chain candidates for the monoclonal sample and 616 for the mixture of 3 mAb sample. The correct chain candidate for the monoclonal sample was ranked #1, despite not having the highest Shotgun-score (267k vs 270k) or MD-score (1815 vs 1818). For the mixture of 3 mAb sample, the chain candidates made up solely out of previously selected FR-CDR-FR candidates were ranked #3-5, with the correct sequence at #5. The top 2 candidates had CDR3 sequences that were previously rejected in the CDR sequencing stage, which were again rejected on the same basis (shorter, less overlapping reads). The isobaric CDR3s still could not be confidently ranked as the scores were too close, with Shotgun-scores between 255.7k and 255.8k and MD-scores between 426.1 and 427.2 (Figure S3). Low fragment coverage combined with other clones being present at similar concentrations seemingly prevented us from resolving the final ambiguities for the mix sample. This is highlighted by the large difference between the MD-scores for the correct chain candidates (426 for the mix sample vs 1815 for the monoclonal sample).

### **Performance on the complex polyclonal samples**

After successfully reconstructing the known sequence of Trastuzumab from the recombinant samples, we proceeded to analyse the polyclonal sample. We selected the most abundant heavy chain (precursor mass 24811.17 Da; Table S1) as a sequencing target and prepared deconvoluted fragmentation spectra from the raw MD-MS data (see **Target mass determination and sample characterization using MD-MS**; Figure 2c). To generate FR candidates for the selected target chain, we submitted *de novo* peptidereads to Stitch (see **Using multi-enzyme shotgun proteomics data for de novo sequencing**). From the *template selection* run we selected IGHV3-33, the most abundant V-region in the Stitch results, for recombination during the *definitive* run (Figure 4a). The Stitch frequency tables from both runs were then converted into FR candidates as described above (see **Framework region sequencing**; Figure S1).

FR candidate generation yielded 8, 2, 384 and 64 candidates for the FR1 to FR4 respectively. After scoring and filtering this was reduced to 2, 1, 3 and 4 candidates (Figure 4b, Table S2). From the FR candidates which remained after the first pass (see **Framework region sequencing**; Table S2), we rejected all but the top scoring candidates with respect to MD-score (10, 155, 163 and 133 for FR1 to FR4 respectively; Figure S3). We then manually selected candidates for each FR based on Shotgun-score. For FR1, we selected the top 2 candidates (34k and 35k Shotgun-score) as the other two candidates had an LTC motif that had a lower Shotgun-score. This left a single ambiguous isobaric residue, an N-terminal pyro-Q/E. For FR2, only 1 candidate had the top MD-score, which was much higher than the alternative candidate (155 vs 105). For FR3 the top 3 Shotgun-score candidates were selected (27k-30k), leaving 2 ambiguous sites (Q/E and TV/RA, Figure 4b). For FR4, the top 4 candidates in terms of Shotgun-score were selected (308k-310k), representing a single ambiguous N-terminal residue (Figure 4b).

Using these FR-candidates, 20, 30 and 120 FR-CDR-FR candidates were generated for CDR1 to CDR3 respectively. The top MD- and Shotgun-scores were unambiguous for CDR1 and CDR2 (Figure S3), identifying the CDR1 as GLTFSTYD (MD-score 118, Shotgun-score 57k), and CDR2 as LWNDGYNK (MD-score 377, Shotgun-score 51k). By selecting these FR-CDR-FR candidates, 2 out of 3 remaining FR3 candidates could be rejected leaving 40 FR-CDR-FR candidates for CDR3. From these, we selected 2 isobaric FR-CDR-FR candidates (***LG***QRR***PL*** and ***GL***QRR***LP***) with the top Shotgun-scores (346.2k and 346.4k) and the second-best MD-score (both 370.7) (Figure 4c, Figure S3).

Recombining the selected FR candidates into chain candidates yielded 975 chain candidates. Two chain candidates were made up of previously selected FR-CDR-FR candidates and scored very well as they had the fourth highest MD-score (434) and top Shotgun-scores (411k; Figure S3). To resolve the remaining ambiguity in the CDR3 (Figure 4d), we revisited the peptide coverage for this region. This revealed a break in the peptide coverage of CDR3 in one of the candidates suggesting the CDR3 sequence LGQRRPL. However, strong support for the LP motif in the CDR3 led us to reinspect the *de novo* reads manually, where we found several reads suggesting the CDR3 sequence LGQRRLP, a sequence absent in any single bridging or overhanging CDR3 reads. Rescoring this sequence indeed revealed an increased Shotgun-score, from 411.3k to 411.7k, providing the final piece of the sequence (Figure 4e).

# Discussion

With this work we show that integration of BU and MD data is beneficial to achieve a higher fidelity for *de novo* extraction of the sequences of antibodies. To provide a solid basis with the *de novo* peptide data, we utilize Stitch (Schulte et al., 2022) although this step still results in errors and leads to ambiguity. To correct the errors and resolve this ambiguity, MD fragmentation data was used. Although the MD data for even the most abundant clone in a mixture is far from complete, we show that it can be used as a potent filter to remove erroneous candidates and even to assist with filling gaps in the sequence. We have used the presented workflow to simultaneously sequence light and heavy chains, but for the sake of brevity have omitted the light chain sequencing efforts in this manuscript. As we analyse one chain at a time, there is little difference between the analysis of light and heavy chains aside from differences arising from the quality of the data or the complexity of the target. Light chains are less complex owing to a lower degree of somatic hypermutation and the lack of a D-segment. Unsurprisingly therefore, these targets performed equally well or better than their heavy chain counterparts.

The polyclonal sample used in this study still represents a non-realistic case for sequencing plasma antibodies, as the sample was dominated by a single clone. However, while moving to more complex samples will surely pose new challenges, it has been shown that circulating antibody repertoires are, more often than previously thought, dominated by a limited number of clones (Bondt, Dingess, et al., 2021; Bondt, Hoek, et al., 2021). We are therefore optimistic that the presented approach will soon be applicable to a significant fraction of polyclonal samples and can be extended to the cases where it falls short. Another point to improve is the need for expert manual interpretation at various points in this workflow, which significantly limits the throughput. Although the main goal of the presented work was to define a broadly applicable protocol for polyclonal antibody sequencing, we have not yet been to define robust score cut-offs for several decision points making this an intermediate step in the development of a fully automated pipeline. The integration of multiple data sources, as well as the diversity of the analysed samples (polyclonal, complex, etc), targets (light or heavy chain, dominant clones, isotypes), regions (FR1-4, CDR1-3) and segments (FRs, FR-CDR-FR, chain), makes this an even bigger challenge. As the field matures however, a point will be reached where scoring functions and corresponding cut-offs can be defined. This will automate an ever-increasing portion of this work, eventually leading to a high throughput, fully automated method.

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Contributions – AJRH conceived the study, BDG performed the data analysis, wrote the required code and prepared the manuscript, RAS supervised the coding and prepared the manuscript, DS prepared the manuscript, AB performed the immunoglobulin capture, ST recorded and analysed the middle-down proteomics data, MH and WP recorded and analysed all shot-gun proteomics data.

Data availability – The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD042757. The software and source code are freely accessible at https://github.com/Bdegraaf1234/FabLabPublic.

# Materials and Methods

## Immunoglobulin capture and Fab generation

### Recombinant IgG1 sample preparation

The IgG purification and generation of IgG1 Fabs for the recombinant monoclonal and mix samples was performed as previously published (Bondt, Hoek, et al., 2021). IgGs were captured using CaptureSelect FcXL affinity matrix (Thermo Scientific). Mobicol spin filters were assembled according to manufacturer instructions and placed in 2 mL Eppendorf tubes. Then 20 µL FcXL affinity matrix slurry was added to the spin filter, followed by three washing steps with 150 µL PBS, in which the liquid was removed by centrifugation for 1 min at 1000 × *g*. Two additional washing steps with 150 µL were performed. The affinity matrix was resuspended in 150 mL PBS, and 100 µg of sample was added. The samples were then incubated while shaking for one hour. Next, the flow-through was collected and the affinity matrix with bound IgGs was washed four times with 150 µL PBS. bound IgGs were digested overnight using 50 µL PBS containing 100 U of the IgdE protease (FabALACTICA®, Genovis, Llund, Sweden) on a thermal shaker (Eppendorf, The Netherlands) at 37 °C. Next, 10 µL of Ni-NTA beads were added to bind and remove the His-tagged protease and left incubating for an additional 30 minutes. The flow through after centrifugation contained the IgG1 Fab fragments generated.

### Serum IgA1 sample preparation

The IgA purification and generation of IgA1 Fabs for the polyclonal sample was performed as previously published (Bondt, Dingess, et al., 2021). IgAs were captured from a patient serum sample using CaptureSelect IgA affinity matrix (Thermo Scientific). 40 µL bead slurry was added directly to Pierce spin columns with screw cap (ThermoFisher Scientific). The beads were then repeatedly washed with 150 µL PBS by centrifugation at 500 × g, room temperature (RT). After the third wash, a plug was inserted to the bottom of the individual spin columns and 100 µL PBS was added to the beads. Twenty microliter of serum was diluted in 150 µL PBS and added, then incubated for 1 hour while shaking. Following the incubation, the plugs were removed from the spin columns and the diluted sample was collected by centrifugation for 1 min at 500 × g, RT. Then the beads were washed four times by addition of 200 µL PBS and subsequent centrifugation for 1 min at 500 × g, RT. After the fourth wash the plugs were reinserted into the bottom of the spin columns. We added to each spin column 50 µL PBS containing 40U SialEXO (SialEXO, Genovis, Llund, Sweden), a sialidase cocktail to remove sialic acids from the O-glycans, and incubated for 1 h at 37°C with continuous shaking at 750 rpm. 1 µL (40 U) of OgpA enzyme (OpeRATOR, Genovis, Llund, Sweden) was then added, and incubation was continued overnight, in and Eppendorf thermal shaker. Next, 10 µL of Ni-NTA beads were added to bind and remove the His-tagged proteases and left incubating for an additional 30 minutes. The flow through after centrifugation contained the IgA1 Fab fragments generated.

## Bottom-up *de novo* sequencing

### Sample preparation

Fab fragments were digested for BU analysis as described previously (Bondt, Hoek, et al., 2021). All purified Fab antibody fragments were dried under vacuum and resuspended in a 50 mM aqueous ammonium bicarbonate buffer. For each bottom-up analysis, 12 µg of sample was used, 3 µg per protease. For digestion with trypsin, chymotrypsin, elastase and thermolysin, a sodium deoxycholate (SDC) buffer was added to a total volume of 80 µL, 200 mM Tris pH 8.5, 10 mM TCEP, 2% (w/v) SDC final concentration. For digestion with pepsin, a urea buffer was added to a total volume of 80 µL, 2M urea, 10 mM TCEP. Samples were denatured for 10 min at 95 °C followed by reduction for 20 min at 37 °C. Next, iodoacetic acid was added to a final concentration of 40 mM and incubated in the dark for 45 min at room temperature for alkylation of free cysteines. Then for trypsin, chymotrypsin and thermolysin, 50 mM ammonium bicarbonate buffer was added to a total volume of 100 µL. For pepsin 1 M HCl was added to a final concentration of 0.04 M. A total of 0.1 µg of each protease was added and the mixture incubated for 4 hours at 37 °C. After digestion 2 µL formic acid was added to precipitate the SDC. SDC was removed by centrifugation for 20 min at maximum speed (20817 × *g*) after which the supernatant was moved to a new tube. The final samples were desalted by Oasis HLB (Oasis).  Sorbent was wetted using 2x 200 µL ACN, followed by equilibration with 2x 200 µL water/10% formic acid.  The sample was loaded and washed with 2x 200 µL Mili Q water/10% formic acid.  Finally, the sample was eluted using 2x 50 µL water/50% ACN/10% formic acid and dried down by vacuum centrifuge. Prior to MS analysis samples were reconstituted in 2% FA.

### LC-MS/MS

Data acquisition was performed on the Orbitrap Fusion Tribrid Mass Spectrometer (Thermo Scientific, San Jose, CA, USA) coupled to UHPLC 1290 system (Agilent Technologies, Santa Clara, CA, USA) as previously published (Bondt, Hoek, et al., 2021). Peptides were trapped (Dr. Maisch Reprosil C18, 3 mm, 2 cm3 100 mm) prior to separation (Agilent Poroshell EC-C18, 2.7 mm, 500 mm 3 75 mm). Trapping was performed for 10 min in solvent A (0.1% HCOOH in Milli-Q), and the gradient was as follows: 0 – 13% solvent B (0.1% HCOOH in 80% CH3CN) over 5 min, 13 – 44% solvent B over 65 min, 44 – 100% solvent B over 4 min, and 100% B for 4 min (flow was split to achieve the final flowrate of approximately 200 nL/min). MS data was collected in a data-dependent fashion with survey scans ranging from 350-2,000 Th (resolution of 60,000 @ m/z 200), and up to 3 sec for precursor selection and fragmentation with either stepped higher-energy collisional dissociation (HCD) set to [25%, 35%, 50%] or electron transfer dissociation (ETD), used with charge-normalized settings and supplemental activation of 27%. The MS2 spectra were recorded at a resolution of 30,000 (@ m/z 200). The AGC targets for both MS and MS2 scans were set to standard within a maximum injection time of 50 and 250 ms, respectively.

### Data analysis

Bottom-up MS/MS spectra were processed with the PEAKS-X *de novo* sequencing suite (Bioinformatics Solutions Inc., Waterloo, ON, Canada). Default settings were used unless explicitly mentioned. Variable modifications were set to pyro-Glu from E, pyro-Glu from Q, oxidation (H/W), oxidation (M). Max 4 variable modifications per peptide, max 5 peptides reported per spectrum, 0.02 fragment mass error tolerance, 20 ppm parent mass tolerance, fixed modification: Carboxymethyl. The resulting *de novo* predictions (referred to as *reads* throughout the manuscript), were inserted into the proteomic short read assembly tool Stitch for two subsequent runs to yield a frequency table and select a germline sequence template for each target chain. The residues of this sequence template were numbered according to the IMGT numbering convention (M. P. Lefranc, 1997). The *de novo* reads were numbered by aligning them to the sequence template using the Smith Waterman algorithm with a custom scoring matrix (Supplementary data 1) and copying the numbering. Throughout the manuscript, AA residues are referred to by their IMGT numbering.

### **Germline database preparation**

The full IMGT database was used as a source of homologous germline sequences (Supplementary data 2). This database was filtered by excluding non-human entries, entries with identical sequences, partial or non-functional entries and sequences including wildcards or non-canonical AAs. The resulting sequences were filtered by selecting one isotype for each entry to create a reduced and nonredundant set of germline template sequences, as described previously (Schulte et al., 2022). Only the constant regions relevant to the analysed sample were included (*i.e.,* IgA1 for the polyclonal sample and IgG1 for the monoclonal and mix samples). These constant regions were cleaved to match the Fab fragments produced by the IgdE and OgpA enzymes. The resulting template sequences were used by Stitch for template selection and read assembly, and to generate the IMGT residue frequency table used for FR generation (Figure S1).

## Middle-down de novo sequencing

### LC-MS/MS

All Fab samples were denatured and reduced in 10 mM tris(2-carboxyethyl)phosphine (TCEP) at 60 °C for 30 min prior to LC-MS/MS analysis. For each LC-MS/MS experiment 2-5 µg of sample was injected. Reversed-phase liquid chromatography was performed by using a Thermo Scientific Vanquish Flex UHPLC instrument, equipped with a 1 mm x 150 mm MAbPac analytical column and directly coupled to an Orbitrap Fusion Lumos Tribrid (Thermo Fisher Scientific, Bremen, Germany). The column preheater, as well as the analytical column chamber, were heated to 80 °C during chromatographic separation.

The recombinant samples were separated over 27 min at a flow rate of 250 µL/min. The polyclonal sample in 22 min at a flow rate of 150 µL/min. Gradient elution was achieved by using two mobile phases A (0.1% HCOOH in Milli-Q HOH) and B (0.1% HCOOH in CH3CN) and ramping up B from 10 to 25% over six and one minute respectively, from 25 to 40% over 14 min, and from 40 to 95% over one minute.MS data were collected with the instrument operating in Intact Protein and Low Pressure mode. The spray voltage was set at 3.3 kV, capillary temperature 350 °C, probe heater temperature 100 °C, sheath gas flow 15, auxiliary gas flow 5, and source-induced dissociation was set at 15 V.

The reduced Fab chains were analysed with a resolution setting of 120k (@ 200 *m/z*) in MS1, which allows for more accurate mass detection of smaller proteins (< 30 kDa) with 250% AGC target and a maximum injection time of 500 ms. For the recombinant samples, 2 µscans were acquired and averaged per MS1 scan, in a range of 500-3000 Th. For the polyclonal sample 5 µscans were averaged in a range of 600-2000 Th. Data-dependent mode was defined as two scans.

MS/MS scans were acquired with a resolution of 120k, a maximum injection time of 500 ms. The ions of interest were mass-selected by quadrupole in a 10, 2 and 4 Th isolation window, for the experiments on the monoclonal, antibody mixture and polyclonal mixture, respectively, and accumulated to the AGC target prior to fragmentation. Electron-transfer dissociation (ETD) was performed using the following settings: 16 ms reaction time, a maximum injection time of 200 ms, and an AGC target of 1e6 for the ETD reagent. For data-dependent MS/MS acquisition, the intensity threshold was set to 5e4. MS/MS scans were recorded in the range of *m/*z = 350-5000 Th using high mass range quadrupole isolation.

### Data analysis

Following the MD LC-MS/MS data acquisition on the Fab fragments,MS1 features were retrieved from the generated RAW files using BioPharmaFinder 3.2 (Thermo Scientific). Deconvolution was performed using the ReSpect algorithm, deconvoluting averaged scans over a selected RT window where the target clone eluted (Table S1). The output mass range for the fragment ions was set at 10 to 40 kDa. Charge states between 10 and 50 were included with a minimum of 6 and 10 adjacent charges for the low and high model mass respectively. No relative abundance or score threshold was used. The target mass was set to 25 kDa, the number of peak models to 1, with a shape of 2 and 2 (left/right). The peak detection minimum significance measure was set to 1 standard deviation and the peak detection quality measured was set to 95%.  The MS2 spectra over the selected retention time were deconvoluted to yield their protonated monoisotopic fragment masses using the Freestyles Xtract algorithm. The minimum charge was set to 1, the maximum charge was set to 50, no thresholds were set for the minimum number of detected charges and the relative abundance.

### Contig scoring and refinement using middle-down data

Throughout the manuscript, we make use of a scoring algorithm to optimize contig placement for a given MD-MS fragmentation spectrum, which we termed the *sliding window* scoring algorithm (Figure S2). This algorithm slides a set of theoretical fragments generated from the provided sequence along a provided *m/z* range, incrementing the fragment masses by a set increment (default 0.01 Th). To limit processing time, peaks in the spectra are binned and the number of non-empty bins are counted for each position. The top scoring positions (default: top 100) are then refined by incrementing by smaller step size while scoring with a more refined scoring function (Olsen & Mann, 2004), and finally the best scoring position is returned. This enables error-tolerant scoring of (sub)sequences, even if the exact prefix- and suffix- mass (distance from the N- and C- termini respectively) is not known, for example for sequence candidates which are not connected to the N- or C- terminus. In addition to a score, it also returns the optimal prefix- and suffix- mass for the sequence, which is used to calculate the theoretical molecular weight of CDRs during CDR sequencing, by calculating the mass gap between adjacent FR candidates.

# References

Aebersold, R., & Mann, M. (2003). Mass spectrometry-based proteomics. *Nature*, *422*(6928), 198–207. https://doi.org/10.1038/nature01511

Aebersold, R., & Mann, M. (2016). Mass-spectrometric exploration of proteome structure and function. *Nature*, *537*(7620), 347–355. https://doi.org/10.1038/nature19949

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). The Generation of Antibody Diversity. In *Molecular Biology of the Cell* (4th ed.). New York (NY): Garland Science.

Altelaar, A. F. M., Munoz, J., & Heck, A. J. R. (2013). Next-generation proteomics: towards an integrative view of proteome dynamics. *Nature Reviews Genetics*, *14*(1), 35–48. https://doi.org/10.1038/nrg3356

Bondt, A., Dingess, K. A., Hoek, M., van Rijswijck, D. M. H., & Heck, A. J. R. (2021). A Direct MS-Based Approach to Profile Human Milk Secretory Immunoglobulin A (IgA1) Reveals Donor-Specific Clonal Repertoires With High Longitudinal Stability. *Frontiers in Immunology*, *12*. https://doi.org/10.3389/FIMMU.2021.789748

Bondt, A., Hoek, M., Tamara, S., de Graaf, B., Peng, W., Schulte, D., … Heck, A. J. R. (2021). Human plasma IgG1 repertoires are simple, unique, and dynamic. *Cell Systems*, *12*(12), 1131-1143.e5. https://doi.org/10.1016/j.cels.2021.08.008

Bornholdt, Z. A., Turner, H. L., Murin, C. D., Li, W., Sok, D., Souders, C. A., … Walker, L. M. (2016). Isolation of potent neutralizing antibodies from a survivor of the 2014 Ebola virus outbreak. *Science*, *351*(6277), 1078–1083. https://doi.org/10.1126/science.aad5788

Briney, B., Inderbitzin, A., Joyce, C., & Burton, D. R. (2019). Commonality despite exceptional diversity in the baseline human antibody repertoire. *Nature*, *566*(7744), 393–397. https://doi.org/10.1038/s41586-019-0879-y

Castellana, N. E., Pham, V., Arnott, D., Lill, J. R., & Bafna, V. (2010). Template Proteogenomics: Sequencing Whole Proteins Using an Imperfect Database. *Molecular & Cellular Proteomics*, *9*(6), 1260–1270. https://doi.org/10.1074/mcp.M900504-MCP200

Charles A Janeway, J., Travers, P., Walport, M., & Shlomchik, M. J. (2001). The generation of diversity in immunoglobulins. In *Immunobiology: The Immune System in Health and Disease* (5th ed.). New York (NY): Garland Science.

Corti, D., Misasi, J., Mulangu, S., Stanley, D. A., Kanekiyo, M., Wollen, S., … Sullivan, N. J. (2016). Protective monotherapy against lethal Ebola virus infection by a potently neutralizing antibody. *Science (New York, N.Y.)*, *351*(6279), 1339–1342. https://doi.org/10.1126/science.aad5224

de Graaf, S. C., Hoek, M., Tamara, S., & Heck, A. J. R. (2022). A perspective toward mass spectrometry-based de novo sequencing of endogenous antibodies. *MAbs*, *14*(1). https://doi.org/10.1080/19420862.2022.2079449

Dupré, M., Duchateau, M., Sternke-Hoffmann, R., Boquoi, A., Malosse, C., Fenk, R., … Chamot-Rooke, J. (2021). De Novo Sequencing of Antibody Light Chain Proteoforms from Patients with Multiple Myeloma. *Analytical Chemistry*, *93*(30), 10627–10634. https://doi.org/10.1021/acs.analchem.1c01955

Guthals, A., Clauser, K. R., & Bandeira, N. (2012). Shotgun Protein Sequencing with Meta-contig Assembly. *Molecular & Cellular Proteomics*, *11*(10), 1084–1096. https://doi.org/10.1074/mcp.M111.015768

Hom, J. R., Tomar, D., & Tipton, C. M. (2022). Exploring the Diversity of the B-Cell Receptor Repertoire Through High-Throughput Sequencing. In J. Rast & K. Buckley (Eds.), *Methods in Molecular Biology: Immune Receptors* (Vol. 2421, pp. 231–241). https://doi.org/10.1007/978-1-0716-1944-5\_16

Johansson, B. P., Shannon, O., & Björck, L. (2008). IdeS: A Bacterial Proteolytic Enzyme with Therapeutic Potential. *PLoS ONE*, *3*(2), e1692. https://doi.org/10.1371/journal.pone.0001692

Kaplon, H., & Reichert, J. M. (2021). Antibodies to watch in 2021. *MAbs*, *13*(1). https://doi.org/10.1080/19420862.2020.1860476

Lefranc, M.-P., & Lefranc, G. (2020). Immunoglobulins or Antibodies: IMGT® Bridging Genes, Structures and Functions. *Biomedicines*, *8*(9), 319. https://doi.org/10.3390/biomedicines8090319

Lefranc, M. P. (1997). Unique database numbering system for immunogenetic analysis. *Immunology Today*, *18*(11), 509. https://doi.org/10.1016/s0167-5699(97)01163-8

Lefranc, M. P. (2003, January 1). IMGT, the international ImMunoGeneTics database®. *Nucleic Acids Research*, Vol. 31, pp. 307–310. https://doi.org/10.1093/nar/gkg085

Ma, B., Zhang, K., Hendrie, C., Liang, C., Li, M., Doherty-Kirby, A., & Lajoie, G. (2003). PEAKS: powerful software for peptide de novo sequencing by tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, *17*(20), 2337–2342. https://doi.org/10.1002/rcm.1196

Marks, C., & Deane, C. M. (2020). How repertoire data are changing antibody science. *The Journal of Biological Chemistry*, *295*(29), 9823–9837. https://doi.org/10.1074/jbc.rev120.010181

Melani, R. D., Srzentić, K., Gerbasi, V. R., McGee, J. P., Huguet, R., Fornelli, L., & Kelleher, N. L. (2019). Direct measurement of light and heavy antibody chains using ion mobility and middle-down mass spectrometry. *MAbs*, *11*(8), 1351. https://doi.org/10.1080/19420862.2019.1668226

Olsen, J. V, & Mann, M. (2004). *Improved peptide identification in proteomics by two consecutive stages of mass spectrometric fragmentation*. *101*.

Peng, W., Pronker, M. F., & Snijder, J. (2021). Mass Spectrometry-Based De Novo Sequencing of Monoclonal Antibodies Using Multiple Proteases and a Dual Fragmentation Scheme. *Journal of Proteome Research*, *20*(7), 3559–3566. https://doi.org/10.1021/acs.jproteome.1c00169

Perez-Riverol, Y., Bai, J., Bandla, C., García-Seisdedos, D., Hewapathirana, S., Kamatchinathan, S., … Vizcaíno, J. A. (2022). The PRIDE database resources in 2022: a hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Research*, *50*(D1), D543–D552. https://doi.org/10.1093/NAR/GKAB1038

Raybould, M. I. J., Marks, C., Lewis, A. P., Shi, J., Bujotzek, A., Taddese, B., & Deane, C. M. (2020). Thera-SAbDab: the Therapeutic Structural Antibody Database. *Nucleic Acids Research*, *48*(D1), D383–D388. https://doi.org/10.1093/nar/gkz827

Schroeder, H. W., & Cavacini, L. (2010). Structure and function of immunoglobulins. *Journal of Allergy and Clinical Immunology*, *125*(2), S41–S52. https://doi.org/10.1016/j.jaci.2009.09.046

Schroeder Jr., H. W. (2006). Similarity and divergence in the development and expression of the mouse and human antibody repertoires. *Dev Comp Immunol*, *30*(1–2), 119–135. https://doi.org/10.1016/j.dci.2005.06.006

Schulte, D., Peng, W., & Snijder, J. (2022). Template-Based Assembly of Proteomic Short Reads For De Novo Antibody Sequencing and Repertoire Profiling. *Analytical Chemistry*, *94*(29), 10391–10399. https://doi.org/10.1021/ACS.ANALCHEM.2C01300

Sen, K. I., Tang, W. H., Nayak, S., Kil, Y. J., Bern, M., Ozoglu, B., … Becker, C. (2017). Automated Antibody De Novo Sequencing and Its Utility in Biopharmaceutical Discovery. *Journal of the American Society for Mass Spectrometry*, *28*(5), 803–810. https://doi.org/10.1007/s13361-016-1580-0

Srzentić, K., Fornelli, L., Tsybin, Y. O., Loo, J. A., Seckler, H., Agar, J. N., … Zhou, M. (2020). Interlaboratory Study for Characterizing Monoclonal Antibodies by Top-Down and Middle-Down Mass Spectrometry. *Journal of the American Society for Mass Spectrometry*, *31*(9), 1783–1802. https://doi.org/10.1021/jasms.0c00036

Toby, T. K., Fornelli, L., & Kelleher, N. L. (2016). Progress in Top-Down Proteomics and the Analysis of Proteoforms. *Annual Review of Analytical Chemistry*, *9*(1), 499–519. https://doi.org/10.1146/annurev-anchem-071015-041550

Tran, N. H., Rahman, M. Z., He, L., Xin, L., Shan, B., & Li, M. (2016). Complete de Novo Assembly of Monoclonal Antibody Sequences. *Scientific Reports*, *6*(31730), 1–10. https://doi.org/10.1038/srep31730

Valgardsdottir, R., Cattaneo, I., Napolitano, G., Raglio, A., Spinelli, O., Salmoiraghi, S., … Golay, J. (2021). Identification of Human SARS-CoV-2 Monoclonal Antibodies from Convalescent Patients Using EBV Immortalization. *Antibodies*, *10*(3), 26. https://doi.org/10.3390/antib10030026