Sequence-to-sequence translation from mass spectra to peptides with a transformer model

Melih Yilmaz*[1], William E. Fondrie*[2], Wout Bittremieux[3], Rowan Nelson[4], Varun Ananth[1], Sewoong Oh[1], and William Stafford Noble[4,1]

[1]Paul G. Allen School of Computer Science and Engineering, University of Washington
[2]Talus Bioscience
[3]Department of Computer Science, University of Antwerp
[4]Department of Genome Sciences, University of Washington

Abstract

A fundamental challenge for any mass spectrometry-based proteomics experiment is the identification of the peptide that generated each acquired tandem mass spectrum. Although approaches that leverage known peptide sequence databases are widely used and effective for well-characterized model organisms, such methods cannot detect unexpected peptides and can be impractical or impossible to apply in some settings. Thus, the ability to assign peptide sequences to the acquired tandem mass spectra without prior information—*de novo* peptide sequencing—is valuable for gaining biological insights for tasks including antibody sequencing, immunopeptidomics, and metaproteomics. Although many methods have been developed to address this *de novo* sequencing problem, it remains an outstanding challenge, in part due to the difficulty of modeling the irregular data structure of tandem mass spectra. Here, we describe Casanovo, a machine learning model that uses a transformer neural network architecture to translate the sequence of peaks in a tandem mass spectrum into the sequence of amino acids that comprise the generating peptide. We train a Casanovo model from 30 million labeled spectra and demonstrate that the model outperforms several state-of-the-art methods on a cross-species benchmark dataset. We also develop a version of Casanovo that is fine-tuned for non-enzymatic peptides. Finally, we demonstrate that Casanovo's superior performance improves the analysis of immunopeptideomics and metaproteomics experiments and allows us to delve deeper into the dark proteome.

Mass spectrometry is currently the most popular analytical technique to characterize the proteome, by identifying and quantifying proteins present in complex biological systems[1]. During a bottom-up mass spectrometry proteomics experiment, proteins from a biological sample are enzymatically digested into peptides, their intact mass and charge are measured, and they are fragmented using tandem mass spectrometry. The fundamental challenge of mass spectrometry proteomics is then to determine the amino acid sequence of the resulting tandem mass (MS/MS) spectra. The standard approach to solve this spectrum identification problem is sequence database searching, during which peptides are simulated *in silico* using known digestion rules from a database of protein sequences potentially present in the biological samples, typically from a reference proteome. Next, each observed MS/MS spectrum is scored against a list of candidate peptides based on simplified peptide fragmentation rules, and the best-scoring peptide-spectrum match (PSM) is reported. Pioneered by the SEQUEST algorithm[2], dozens of database search engines have been subsequently developed and are very widely deployed[3].

However, a fundamental requirement for sequence database searching is that the set of proteins that may be present in the sample is known in advance. While this is often the case for samples generated from species with well-characterized genomes, relying on a database prevents the detection of unexpected peptides. Such unexpected peptides include not just peptides derived from contaminant proteins or that arise due to variability in sample processing[4], but also biologically and clinically relevant peptides, such as peptides that

*Equal contributions

deviate from the reference proteome due to genetic variation, peptides with unexpected post-translational modifications (PTMs), and peptides originating from foreign sources, such as microbes or consumed foods. Furthermore, there are tasks where generating a peptide database can prove impractical or even impossible. For example, the antigenic peptides presented by major histocompatibility complex (MHC) proteins—the "immunopeptidome"—are often generated from their parent proteins in an unpredictable manner, requiring at minimum every possible protein subsequence to be considered[5–7]. Similarly, constructing a peptide database for antibody sequencing is nearly impossible, due to the sequence variants created by V(D)J recombination[8]. Finally, creating an accurate database for mixtures of many organisms—metaproteomics—such as from microbiome or environmental samples, is often not feasible[9].

Such applications require the ability to sequence peptides directly from the acquired MS/MS spectra *de novo*. Early *de novo* peptide sequencing algorithms used heuristic search[10] or dynamic programming[11,12] to propose peptides for the observed MS/MS spectra. In addition to dynamic programming, the PepNovo algorithm[13] attempted to account for rules governing peptide fragmentation in its probabilistic score function and is closely related to the hidden Markov model employed by Fischer *et al.*[14] In 2015, the Novor algorithm[15] improved the state of the art by using a decision tree as the score function for its dynamic programming algorithm.

More recently, as in many other fields, deep learning has become the preferred solution for *de novo* peptide sequencing. DeepNovo[8] combines a convolutional neural network and a recurrent neural network to predict the subsequent amino acid when provided an MS/MS spectrum and a peptide prefix. SMSNet[16] uses a similar network architecture but reconciles the predicted sequences against a user-supplied peptide database. PointNovo[17], the successor to DeepNovo, leverages an order-invariant network architecture to model high-resolution MS/MS spectra[18]. Finally, pNovo 3[19] first generates candidate peptides for each MS/MS spectrum using dynamic programming, after which a final score is determined by matching the spectrum against a theoretical spectrum for each candidate peptide, simulated using the pDeep[20] learning-to-rank framework.

Despite the advances of these deep learning-based methods for *de novo* peptide sequencing, they still suffer from several limitations. *De novo* tools typically can only annotate a minority of MS/MS spectra compared to sequence database searching, they struggle with natively encoding high-resolution MS/MS data, and they employ complex neural network architectures and post-processing steps.

To address these issues, here we describe Casanovo, which reframes the *de novo* peptide sequencing task as a machine translation problem: like translating a sequence of words in a sentence from one language to another, Casanovo translates a sequence of peaks in an MS/MS spectrum into a sequence of amino acids of the generating peptide. To do so, we leverage the state-of-the-art architecture for modeling natural language—the transformer[21]. The transformer architecture allows Casanovo to directly use the m/z and intensity value pairs that comprise an MS/MS spectrum without discretization of the m/z axis and to directly output a predicted peptide sequence without a complicated dynamic programming step. We have previously[22] trained Casanovo on a limited collection of mass spectra from the multi-species benchmark used by Tran *et al.*[8] In this work, we present significant improvements to Casanovo and demonstrate its effectiveness at tackling common challenges with *de novo* peptide sequencing. We expand our training set to use 30 million confident PSMs from the MassIVE-KB spectral library[23], and we add a beam search decoding procedure to predict the best peptide for each MS/MS spectrum. We demonstrate that, together, these updates significantly improve Casanovo's already state-of-the-art performance. Additionally, we fine-tune a non-enzymatic version of Casanovo for tasks such as immunopeptidomics. We demonstrate how high-performance *de novo* peptide sequencing using Casanovo enables fast and effective immunopeptidome analysis, bolsters the characterization of metaproteomes, and sheds light on the dark proteome.

Results

A transformer architecture enables processing of raw mass spectra

Casanovo uses a transformer architecture to perform a sequence-to-sequence modeling task, from MS/MS spectrum to the generating peptide (Figure 1). Transformers are built upon the attention function[21], which allows transformer models to contextualize the elements of a sequence; transformer models thus learn the relationships of sequence elements to one another and how their interaction should be interpreted. As such,

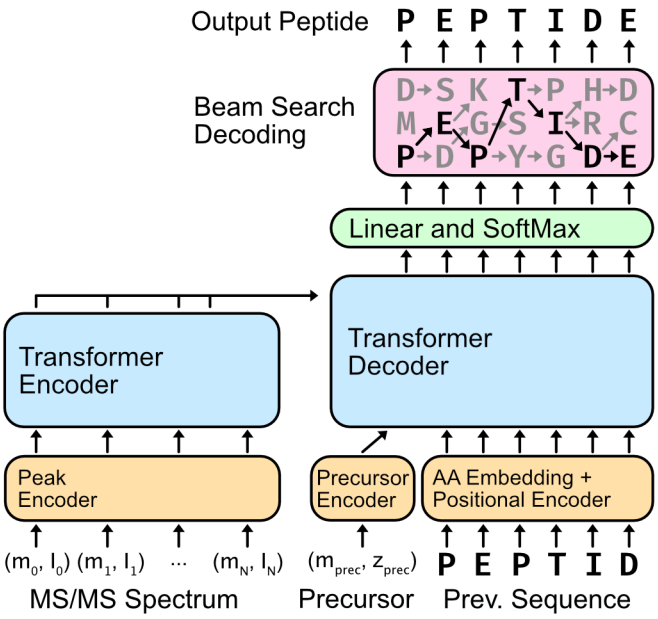


Figure 1: **Casanovo performs *de novo* peptide sequencing using a transformer architecture.** The peaks from each MS/MS spectrum are contextualized by the transformer encoder. The resulting peak encodings are then fed into the transformer decoder along with the observed precursor mass and charge to iteratively decode the peptide sequence. Casanovo uses a beam search decoding strategy, following the most promising sequence predictions until they terminate or exceed the precursor mass. The highest scoring peptide sequence is returned as the putative peptide that generated the MS/MS spectrum.

the transformer architecture has found success in not only natural language processing, but also applications to biological sequences[24,25].

In Casanovo, each peak in an observed MS/MS spectrum is considered as an element in a variable length sequence. The m/z and intensity values of each peak are encoded using, respectively, a collection of sinusoidal functions and a learned linear layer, and these encodings are summed. The encoded peaks are then input into the transformer encoder, where context is learned between pairs of peaks in the MS/MS spectrum. The contextualized peak encodings are then used as input to the transformer decoder for predicting the peptide sequence.

The process of decoding proceeds in an iterative, autoregressive manner. We begin by providing the mass and charge of the observed precursor. The transformer decoder uses the contextualized peak encodings and the precursor information to begin predicting amino acids of the peptide. With the first predicted amino acid, we retain the top k residues, where k is a user-selected value for the number of beams in our beam search. In each subsequent iteration, amino acids are added to the decoded peptide sequence, retaining the top k sequences until the decoded sequences for all of the beams have terminated or exceeded the precursor mass. Finally, the sequence with the highest score is retained as the putative peptide that generated the provided MS/MS spectrum.

In generating its predictions, Casanovo will inevitably fail to generate plausible peptides for some MS/MS spectra. For example, some MS/MS spectra contain too few fragment ions to be sequenced reliably, or the true generating peptide may bear a modification that is unknown to Casanovo. We therefore refine the PSMs proposed by Casanovo using a simple precursor mass filter: any PSMs for which the m/z of the peptide falls outside the specified tolerance of the observed precursor, including potential isotopes, is discarded. This filter eliminates many poorly scored peptides from consideration. In our evaluations, PSMs that would normally be removed by this filter were retained and ranked last among all PSMs assigned by Casanovo.

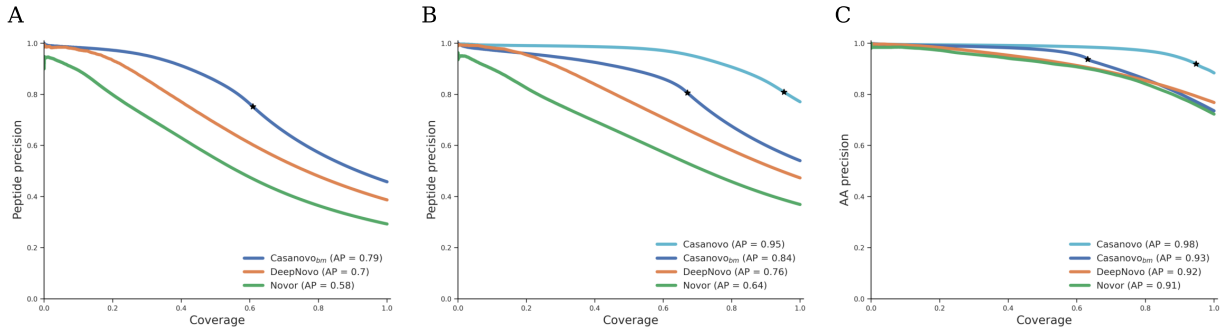


Figure 2: **Casanovo outperforms DeepNovo and Novor on a nine-species benchmark.** (A) Casanovo maintains high peptide-level precision (the proportion of correctly predicted peptides) across all values of coverage (the proportion of spectra for which a prediction is made). Each curve is computed by sorting predicted peptides for all nine species according to their peptide-level confidence scores. For Casanovo, all peptides that pass the precursor m/z filter are ranked above peptides that do not pass the filter, and the boundary is indicated by a star on each curve. (B) Same as panel (A), but using the revised benchmark and including a version of Casanovo trained on MassIVE-KB. (C) Casanovo's amino acid-level precision is greatly improved by the expanded training data provided by MassIVE-KB. The test set is the revised nine-species benchmark, with PSMs only containing modifications considered by both DeepNovo and Casanovo.

Casanovo outperforms state-of-the-art methods

To evaluate Casanovo, we first used the nine-species benchmark dataset originally created by Tran *et al.*[8] to compare the performance of three *de novo* peptide sequencing algorithms: Novor, DeepNovo, and Casanovo. For these comparisons, we used the publicly available, pretrained version of Novor and used it to sequence the MS/MS spectra in the benchmark dataset. DeepNovo and Casanovo were trained in a cross-validated fashion, systematically training on eight species and testing on the remaining species. For DeepNovo, we used the models trained and provided by Tran *et al.*[8] for each of the cross-validation splits. This benchmark version of Casanovo, Casanovo$_{bm}$, employs a simple greedy decoding algorithm, rather than beam-search decoding. The results (Figure 2A) revealed that Casanovo$_{bm}$ substantially improved peptide-level sequencing performance over Novor and DeepNovo, with an average precision of 0.790 for Casaonovo$_{bm}$ compared to 0.578 and 0.697 for Novor and DeepNovo, respectively. These results are consistent across all nine species in the benchmark dataset (Supplementary Figure S1).

We hypothesized that Casanovo could achieve even better performance if provided more data from which to learn; hence, we turned to the MassIVE-KB spectral library of human MS/MS proteomics data[23]. MassIVE-KB provided us with a set of 30 million high confidence PSMs, which we previously collected for training our GLEAMS embedding model [26]. This dataset contains not only a greater diversity of pepides and MS/MS spectra generated from multiple instruments, but also additional types of post-translational modifications. We therefore created a new version of the nine-species benchmark dataset using the same nine PRIDE datasets but including seven different types of variable modifications (methionine oxidation, asparagine deamidation, glutamine deamidation, N-terminal acetylation, N-terminal carbamylation, N-terminal NH_3 loss, and the combination of N-terminal carbamylation and NH_3 loss). In the process, we also fixed several problems that we uncovered in the previous benchmark, including adding consideration of isotope errors and eliminating peptides that occur in multiple species (see Methods for details). The final, revised benchmark dataset consists of 2.8 million PSMs drawn from 343 RAW files.

The results from evaluating with respect to this revised benchmark demonstrate the value of training from a much larger collection of PSMs (Figure 2B). With a larger training set, the average precision of Casanovo increases from 0.84 to 0.95. Furthermore, Casanovo succeeds in making a larger proportion of predictions with m/z values that fall within 30 ppm of the observed precursor (signified by the location of the stars in Figure 2B), increasing from 67% to 94%. This version of Casanovo incorporates beam-search decoding, which improves both average precision and coverage compared to greedy decoding for the same

model (Supplementary Figure S3).

In one sense, this comparison is unfair because some of the spectra in the new version of the benchmark contain PTMs that cannot be identified by some of the competing methods. We therefore eliminated these spectra from each test set and then re-computed the precision-coverage curve. The results (Supplementary Figure S4) are largely unchanged, suggesting that the PTMs contribute little to the observed overall differences in performance.

In addition to evaluating Casanovo's ability to correctly predict whole peptides, we also evaluated Casanovo's ability to the predict individual amino acids of each peptide. We did so by ranking amino acids by their associated confidence score and then plotting a precision-coverage curve. We compared two versions of Casanovo (trained from the first benchmark and from MassIVE-KB) with DeepNovo on the revised nine-species benchmark with new modifications eliminated (Figure 2C). The amino acid-level performance was consistent with the trends we observed in peptide-level performance, with Casanovo outperforming Novor and DeepNovo: Casanovo trained on MassIVE-KB achieves a remarkable average precision of 0.98.

Casanovo unravels the immunopeptidome

One important application of *de novo* peptide sequencing is the characterization of the peptides presented by major histocompabilitility complexes (MHCs), which is commonly referred to as the "immunopeptidome." These antigen peptides are presented on the extracellular surface and serve as targets for immune cell recognition. However, because these antigen peptides are generated through lysosomal or proteasomal degradation, they do not exhibit the characteristic tryptic termini from most proteomics experiments. Consequently, the peptide search space is exponentially larger than considering only tryptic peptides—every peptide subsequence in a protein within a defined peptide length must be considered. Furthermore, mutations in these peptides are of particular interest, because these mutation-containing neoantigens may serve as tumor-specific markers to activate T cells and initiate antitumor immune responses. Unfortunately, expanding the search space to consider all possible mutated peptides is prohibitive both in terms of search speed and statistical power for traditional proteomics search engines.

Although immunopeptidomics is a prime application for *de novo* sequencing, naively applying Casanovo directly to immunopeptidomics data is problematic: the standard Casanovo model is heavily biased to predict tryptic peptides due to their overrepresentation in MassIVE-KB. To demonstrate this effect, we analyzed five mass spectrometry runs generated from MHC class I peptides isolated from MDA-MB-231 breast cancer cells[5] in two different ways: first using Casanovo and second by searching against a non-enyzmatic digestion of the human proteome (see Methods). Among the peptides accepted at 1% FDR by the database search procedure, we observed a low proportion of "tryptic" peptides, i.e., peptides with C-terminal amino acids of K (1.12%) or R (0.80%). In contrast, among the top-scoring 10% of the Casanovo predictions, we observed a greater than six-fold increase in the rate of tryptic peptide predictions (5.87% K and 6.76% R).

We hypothesized that we could reduce this tryptic bias and produce a version of Casanovo that is better suited to immunopeptidomics data by fine tuning our existing model using data that lacks a tryptic bias. To create such a dataset, we combined data from two sources. First, we segregated PSMs from MassIVE-KB according to their C-terminal amino acid and then uniformally sampled up to 50,000 peptides within each group. For most amino acids, MassIVE-KB contained fewer than 50,000 PSMs, so for these we supplemented by randomly extracting additional PSMs from the PROSPECT collection[27] (Supplementary Table S1). We then split this new collection of 1 million PSMs into training, validation, and testing sets. We then fine-tuned our existing Casanovo model by training it until convergence on this non-enzymatic training set.

The resulting model, Casanovo$_{ne}$, performs markedly better than the original Casanovo model at predicting peptides in our held-out, non-enzymatic test set. On the held-out test set of 100,000 non-enzymatic PSMs, Casanovo$_{ne}$ achieves an average precision of 0.663, compared with 0.461 for the original Casanovo model on the same data (Figure 3A). The predicted C-terminal amino acid frequencies are also much closer to the true frequencies, with K and R dropping to 1.11% and 1.01%, respectively (Figure 3B).

We next used Casanovo$_{ne}$ to sequence the immunopeptidome of MDA-MB-231 breast cancer cells[5]. For each peptide predicted by Casanovo, we investigated whether it (1) occurs anywhere within the human proteome, and (2) occurs within the set of peptides detected using a database search procedure. We first searched the data against a non-enzymatic digestion of the human proteome using the Tide search engine[28] followed by Percolator post-processing[29], using settings similar to those in the original study[5] (see Methods).

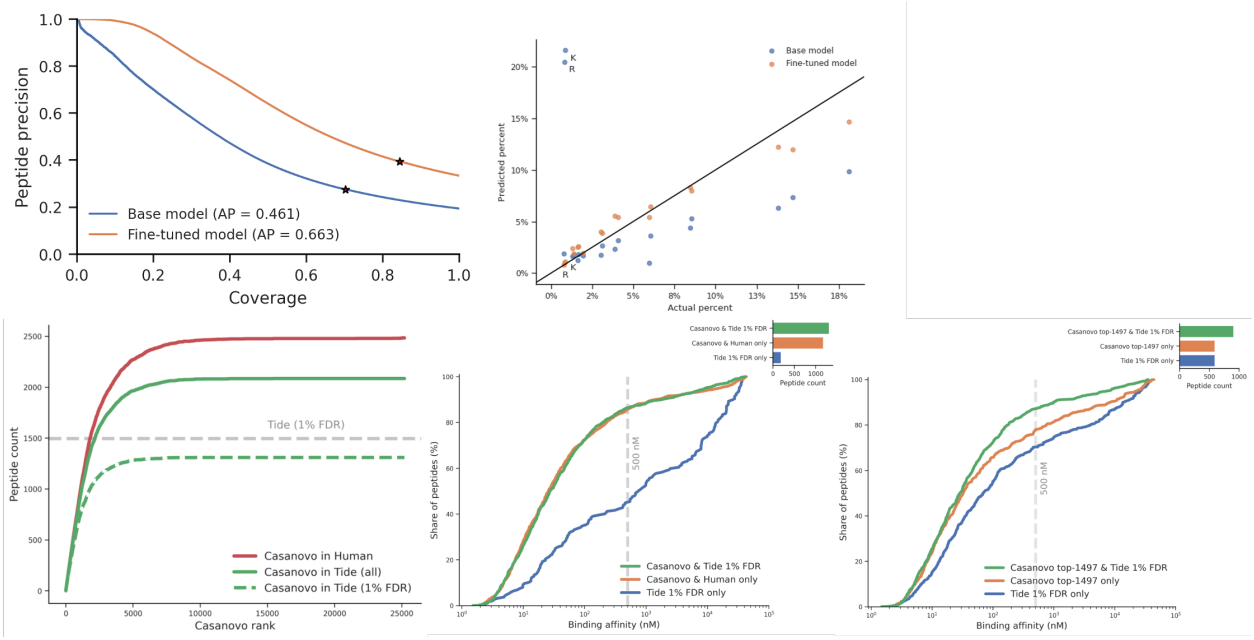


Figure 3: **Fine-tuning reduces Casanovo's bias for tryptic peptides.** (A) Fine-tuning Casanovo (Casanovo$_{ne}$) improves peptide-level precision on sequencing MS/MS spectra generated by non-tryptic peptides. (B) Casanovo$_{ne}$ predicts non-tryptic C-terminal peptides more readily than the standard Casanovo model, improving performance on the non-enzymatic validation set. (C) Casanovo detects many peptides that are present in the human proteome but are not detected via database search. The dashed line only includes peptides detected by database search within the 1% FDR threshold, whereas the solid green line includes all peptides from the database search, irrespective of FDR threshold. (D) The peptides proposed by Casanovo generally have higher predicted binding affinities for the MHC class I receptor, matching the performance of a Tide database search. The vertical bar corresponds to the 500 nM binding affinity below which peptides are predicted to be MHC binders. (E) Similar to panel (D), but considering only the 1497 peptides that are accepted at 1% FDR by Tide and a corresponding set of 1497 highest-confidence Casanovo peptides.

Out of 25,195 unique peptides predicted by Casanovo, 2485 match to the human proteome, and a majority of these overlap with the 1497 unique peptides identified by Tide at 1% FDR. Notably, these overlapping peptides are predicted with high confidence by Casanovo, almost all within the first 10,000 Casanovo predictions (Figure 3C). Casanovo predicts an additional 1175 peptides that match to the human proteome but are not identified by Tide at 1% FDR, and further analysis shows that 776 (66.0%) of these peptides correspond to Tide hits that were not accepted at the 1% FDR threshold.

To further investigate the plausibility of Casanovo predicted peptides as MHC antigens, we used NetMHCpan-4.1[30] to predict MHC binding affinity for these peptides. First, we compared peptides that were identified by both Casanovo and database search with peptides that were predicted only by Casanovo and match to the human proteome. These two groups exhibit similar distributions of predicted binding affinity profiles, with 86% of peptides are predicted to be MHC binders at 500 nM (Figure 3D). In contrast, when we evaluate peptides that are identified by database search but not by Casanovo, the proportion of predicted MHC binders drops substantially to 45%. Overall, these results suggest that Casanovo not only identifies more peptides matching to the human proteome than the standard database search procedure, but the peptides Casanovo predicts are also more likely to bind MHC antigens.

We also explored an alternative method for comparing Casanovo and Tide results, which does not rely on mapping Casanovo predictions to the reference proteome. For this analysis, we consider the 1497 peptides identified by Tide at 1% FDR alongside the top 1497 highest confidence Casanovo predictions. The results (Figure 3E) agree with those in Figure 3D: the 906 peptides in common between the two sets of peptides achieve the highest proportion of MHC binders (87%), the Casanovo-only predictions achieve a slightly lower percentage (78%), and the Tide-only predictions have the lowest percentage of MHC binders (70%).

Casanovo accurately sequences peptides from complex metaproteomes

Proteomics applications extend far beyond the analysis of single model organisms or well-characterized biological systems. Indeed, there is growing interest in using mass spectrometry proteomics methods to investigate the dynamics of complex biological ecosystems—whether microbiomes or environmental specimens—for which the identities of its members cannot be known *a priori*. Due to the unknown complexity of the sample and even the lack of reliable reference proteomes for the likely species in the sample, these metaproteomics experiments are difficult to analyze. One solution to these problems is to search the spectra against a large database, such as one containing all the microbial sequences in public databases for a sample that is likely dominated by unknown microbes. This "big database" approach is widely used but suffers from a significant loss in statistical power due to the implicit multiple hypothesis testing correction that must be made to account for the size of the database. An alternative solution involves first subjecting the sample to genome sequencing, and then using the inferred peptide sequences as the basis for a "metapeptide" database. This approach yields better power to detect peptides[31] but requires the availability of a matched DNA sample and the additional cost associated with DNA sequencing.

We hypothesized that Casanovo's improved *de novo* sequencing capabilities would be useful in both scenarios—either in the presence or absence of a metapeptide database. To test this hypothesis, we applied Casanovo to data from six previously published ocean metaproteomics samples, three from the Bering Strait and three from the Chukchi Sea[31]. Critically, these samples were also subjected to DNA sequencing; hence, in addition to the non-redundant environmental database, we also have a metapeptide database for each sampling location.

We began by measuring the extent to which peptides detected by Casanovo occur within the corresponding metapeptide database or within the larger, non-redundant protein database. To control the error rate for the matching of Casanovo predictions to these databases, we employed a procedure similar to target-decoy competition used in the false discovery rate estimation for database search (see Methods for details) and only considered as correct Casanovo peptides found in the corresponding database that fall within the 1% random matching threshold. Using this logic, we obtain much better power to detect peptides using Casanovo than using a standard database search procedure against the metapeptide database (Figure 4A–B). In particular, when we search the data against the metapeptide database using Tide[28] followed by Percolator[29], we detect 5623 peptides at 1% FDR in the Bering Strait data and 2460 peptides in the Chukchi Sea data. In contrast, if we run Casanovo and accept as correct only peptides that appear in the metapeptide database (subject to our 1% random matching criterion), then we detect 7798 and 3447 peptides, respectively, in the two datasets,

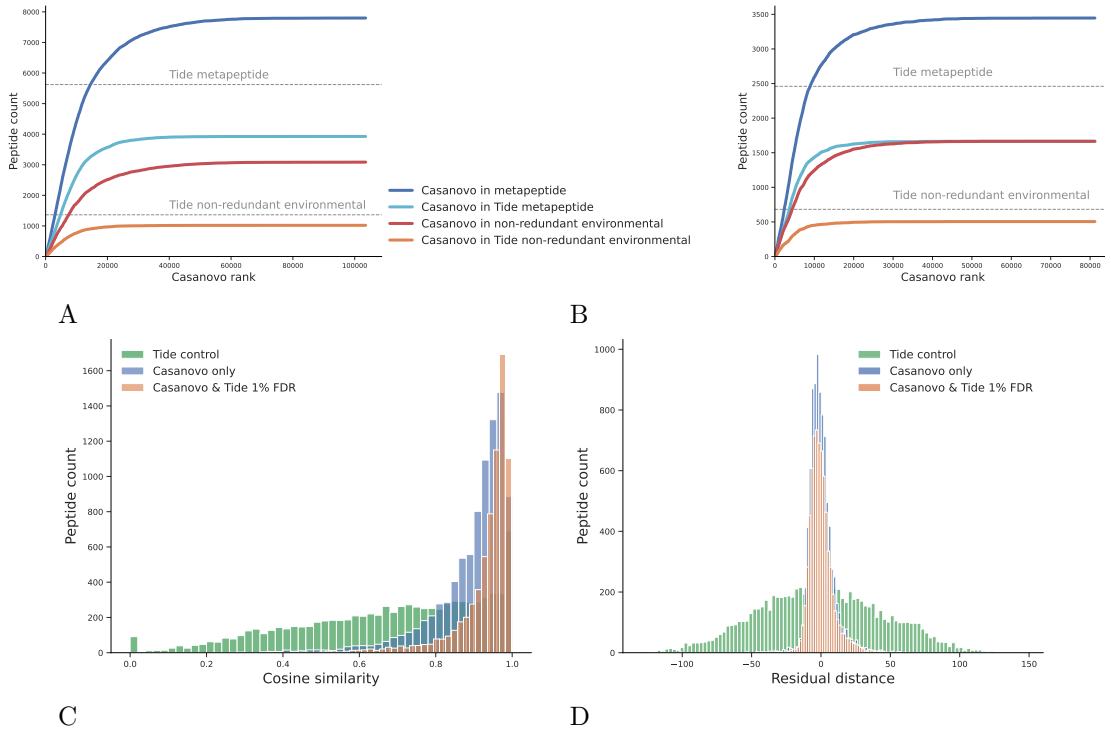


Figure 4: **Casanovo improves power to detect peptides from metaproteomics samples.** Casanovo assigns more peptides matching the metapeptide database and the non-redundant environment database than Tide and Percolator at 1% FDR in seawater samples from (A) the Bering Sea and (B) the Chukchi Sea. Peptides are ranked according to the Casanovo confidence score, assigning each peptide the maximum score across all three runs from each sampling location. Horizontal lines indicate the total number of distinct peptides detected by Tide+Percolator, searching against two different databases. (C) The PSMs assigned by Casanovo at a 1% error rate and Tide and Percolator at 1% FDR have high cosine similarities to the predicted MS/MS spectra for the respective peptides from Prosit when compared to control PSMs sampled from the Tide search results with >10% FDR. Each group represents the aggregated results for Bering and Chukchi Sea data, as well as non-redundant environmental and metapeptide databases. (D) The PSMs assigned by Casanovo at a 1% error rate and Tide and Percolator at 1% FDR closely align with the predicted retention times from Prosit.

representing increases of 39% and 40%. Casanovo also outperforms database search when we consider the non-redundant protein database rather than the sample-specific metapeptide database. We detect 1364 peptides in the Bering Strait data and 682 peptides in the Chukchi Sea data at 1% FDR by searching the non-redundant environmental database using Tide and Percolator. In comparison, Casanovo predictions, filtered at 1% error rate using the environmental database, detect 3086 and 1665 peptides, respectively, representing increases of 125% and 144%, respectively.

To validate the peptides that were detected by Casanovo but not the database search, we used the Prosit machine learning tool[32] to predict spectrum peak intensities and retention times for peptide identifications. First, we compared the cosine similarities between the observed and predicted MS/MS spectrum peak intensities across three groups of peptides: peptides only predicted by Casanovo that matched to the database with 1% error, peptides detected both by Casanovo and by Tide and Percolator at 1% FDR, and a control group of peptides detected by Tide and Percolator with >10% FDR. The control group was randomly sampled to be the same size as the Casanovo-only group. The results (Figure 4C) indicate that the Casanovo-only identifications have a high concentration of high cosine similarity peptides, similar to the overlapping identifications between Casanovo and database search. This stands in contrast with the control group, which exhibits a much broader distribution of cosine similarities.

Second, we compared the observed retention times with the predicted retention times from Prosit for the same three groups of peptides. For each group, we calibrated the predicted retentions times to the observed retention times using linear regression (Supplementary Figure S6). We observed that the peptides detected only by Casanovo and those detected by Casanovo and Tide had a similar slope and resulted in similar residual distributions (Figure 4D). When compared against the control group, the residual distributions for peptides only detected by Casanovo and those detected by Casanovo and Tide are close to zero.

Ultimately, Casanovo does not yet allow us to achieve as much power with the non-redundant database as with the metapeptide database. For example, for the Bering Strait data, the union of the 3086 peptides detected using Casanovo and the 1364 peptides detected using database search is 3430, which is fewer than the 5623 peptides detected using the metapeptide database. (The corresponding numbers for the Chukchi Sea data are 1842 and 2460.) This difference is perhaps not surprising, because the environmental non-redundant database is incomplete: 3715 of the 5623 peptides found by the database search procedure in the Bering Strait metapeptide database are not even present in the environmental database. Thus, a rigorous FDR control procedure for *de novo* peptide sequencing is needed in order to rescue the many peptides that are correctly detected by Casanovo but cannot be validated by matching to a database.

Casanovo shines a light on the dark proteome

The "dark matter" of mass spectrometry-based proteomics consists of MS/MS spectra that are observed repeatedly across experiments but consistently fail to be identified. In many cases, these MS/MS spectra may have been generated by peptides that are not in the canonical human proteome, because they represent contaminant peptides, result from non-standard enzymatic cleavage, or contain sequence variants. We hypothesized that Casanovo could shed light on some of this dark matter.

Accordingly, we applied Casanovo to a collection of MS/MS spectra drawn from a previous analysis,[26] in which 511 million human spectra from MassIVE were grouped into 60 million clusters, and the clusters were systematically analyzed using targeted open modification searching of representative spectra. The analysis yielded a collection of 39 million unidentified clusters, containing a total of 207 million MS/MS spectra. For our analysis, we selected 3.4 million of these unidentified, clustered MS/MS spectra from eight randomly selected MassIVE datasets. These MS/MS spectra belong to 573,597 distinct clusters. We used the non-enzymatic Casanovo model (Casanovo$_{ne}$) to assign a peptide to each selected MS/MS spectrum, eliminating peptides for which the predicted m/z falls outside the associated mass range, yielding a total of 1.3 million predicted peptides.

We sought to ascertain how well Casanovo had assigned peptide sequences to these dark matter clusters by addressing this question in two complementary ways. First, we identified all clusters in which a plurality (and at least two) of the spectra were assigned to the same peptide sequence, and then we mapped those peptides to the human reference proteome, allowing at most one amino acid mismatch. The first step of this procedure assigns peptides to 90,413 (16%) of the clusters, of which 65% could be matched to the human proteome. The clusters identified in this fashion vary in size, ranging from 2 to 542 spectra per

cluster, but when we limited the above analysis only to clusters larger than a certain size, we observed that the shares of identified clusters more than doubled (Supplementary Figure S7). Second, we performed a complementary analysis, first eliminating all predicted peptides that do not occur within the human proteome (again, allowing one mismatch) and then finding clusters with two or more spectra assigned the same sequence and no other spectrum assigned to a different sequence. This procedure assigns peptides to 52,786 clusters, corresponding to 9% of all previously unidentified clusters. The overlap between the two approaches—plurality vote followed by proteome matching or vice versa—is high: 98% of the 52,786 clusters overlapped with the clusters from the previous analysis. Overall, Casanovo is able to assign peptides to 196,084 of the 3.4 million unidentified MS/MS spectra using the combination of these two strategies.

One potential reason for an MS/MS spectrum to remain unidentified is the presence in the generating peptide sequence of a genetic variant that does not appear in the reference proteome. To investigate whether Casanovo is identifying such sequences, we looked more closely at the subset of Casanovo cluster assignments that match to the human proteome with a single amino acid mismatch, focusing on the 51,857 assignments that agree between the two methods described above. Two pieces of evidence suggests that these peptides are indeed enriched for genetic variants. First, we observe an enrichment for amino acid substitutions that can be explained by a corresponding single-nucleotide substitution. Among the Casanovo predictions, 83.4% correspond to a potential single-nucleotide substitution, compared with only 38.6% of all possible amino acid substitutions that fit this criterion. Second, we see a strong enrichment for substitutions with positive BLOSUM62 scores[33]. The BLOSUM score is an integerized log-odds score indicating the empirical substitutability of one amino acid for another. In the BLOSUM62 matrix, only 11% of the 380 non-diagonal entries are positive. However, if we rank the Casanovo-predicted substitutions by frequency, we find that the top five substitutions have BLOSUM scores of 1 or 2 (Supplementary Table S3). This observation strongly suggests that the Casanovo is predicting substitutions that are biochemically plausible.

Discussion

Casanovo's excellent performance derives from two sources: the availability of a very large set of training data, and the use of a machine learning architecture that can make use of that data. For the former, we trained Casanovo from the existing MassIVE-KB resource, which is limited to human data. The field as a whole would benefit from additional efforts at systematically annotating more diverse datasets for the purposes of training large-scale machine learning models. The transformer architecture is uniquely suited to contextualize the elements of variable length sequences and has therefore proven immensely successful in modeling natural language. In comparison to recurrent neural networks, the transformer architecture is able to learn long-range dependencies between the elements of a sequence and can be parallelized for efficient training. By encoding the peaks of a mass spectrum as a sequence, similar to tokenizing the words of a sentence, Casanovo leverages the strengths of the transformer architecture and the rapid advances pioneered in large language models to improve *de novo* peptide sequencing from MS/MS spectra.

Casanovo's utility extends beyond the applications we have demonstrated here. Most obviously, any application in which a peptide database is unavailable, incomplete, or extremely large may benefit from *de novo* sequencing, such as paleoproteomics, forensics, or astrobiology[34]. However, even in the analysis of human or model organism data, Casanovo can assist in the detection of "foreign" spectra, i.e., spectra generated by peptides that are not present in the database. Such foreign spectra might correspond to contaminants introduced during the experiment itself, but they can also represent microbial species, genetic variation, or trans-spliced peptides. In general, we envision applying Casanovo as a post-processor for spectra that fail to be assigned a peptide during a standard database search procedure, akin to the last stage of a cascade search[35].

One important application of *de novo* sequencing that we have not yet explored is antibody sequencing. However, a recent publication carried out a systematic comparison of six *de novo* sequencing tools, including Casanovo, on the problem of antibody sequencing[36]. The results show that Casanovo strongly outperforms the competing methods by all of the measures that the authors considered. Notably, this comparison employed a version of Casanovo that used greedy decoding and was trained on only 2 million spectra. Hence, our results (Figure 2B) suggest that the version of Casanovo trained from 30 million spectra will yield even better antibody sequencing performance.

We anticipate many opportunities for fine-tuning the Casanovo model for particular applications. Our analysis with the non-enzymatic model suggests that Casanovo's enzymatic bias can be adjusted by using a relatively small amount of training data. Thus, in the short term, we plan to train variants of Casanovo that are appropriate for a variety of different cleavage enzymes. The Casanovo software makes such fine-tuning straightforward, so any user interested in adapting the model to a particular experimental setting should be able to do so. Longer term, an ideal model would take as input a spectrum along with relevant metadata, such as the digestion enzyme, collision energy, and instrument type, and predict accurately for many different types of experimental settings.

Clearly, at this stage, one of the key bottlenecks in the field is the absence of a rigorous method for confidence estimation for *de novo* sequencing. In our metaproteomics analysis, we have matched Casanovo predictions to a target and corresponding decoy peptide database, but such an approach misses out on the power of *de novo* sequencing to assign peptides to foreign spectra. Thus, an open question is whether, for a given data-dependent acquisition dataset, Casanovo outperforms a standard database search procedure in terms of statistical power to detect peptides. Trained from sufficiently large training sets, we may be approaching the end of database searching as the go-to method for analysis of DDA tandem mass spectrometry data.

Methods

Casanovo

Casanovo consists of a transformer encoder and decoder stack as described by Vaswani *et al.*[21], which are respectively responsible for learning latent representations of the input spectrum peaks and decoding the amino acid sequence of the spectrum's generating peptide. The encoder takes d-dimensional spectrum peak embeddings as input and outputs d-dimensional latent representation vectors for each peak. Subsequently, the decoder takes as input these representations of prefix amino acids, coupled with a d-dimensional precursor embedding encapsulating precursor m/z and charge information, to predict the next amino acid in the peptide sequence. We discuss different aspects of our modeling strategy in detail below.

Input embeddings

Each spectrum $S = \{(m_j, I_j)\}_{j=1}^{N}$ is a bag of peaks, where each peak (m_j, I_j) is a 2-tuple representing the m/z value and intensity of the peak. The m/z value and intensity are embedded separately before being summed to yield the input peak embedding. We use a fixed, sinusoidal embedding[21] to project each m/z value to a d-dimensional vector, the m/z embedding f. Specifically, we create the m/z embedding from an equal number of sine and cosine waveforms spanning the wavelengths from 0.001 to 10,000 m/z, where each feature in the embedding f_i is

$$f_i = \begin{cases} \sin(m_j/(\frac{\lambda_{\max}}{\lambda_{\min}}(\frac{\lambda_{\min}}{2\pi})^{2i/d})), & \text{for } i \leq d/2 \\ \cos(m_j/(\frac{\lambda_{\max}}{\lambda_{\min}}(\frac{\lambda_{\min}}{2\pi})^{2i/d})), & \text{for } i > d/2 \end{cases} \tag{1}$$

where $\lambda_{\max} = 10,000$ and $\lambda_{\min} = 0.001$. These input embeddings provide a granular representation of high-precision m/z information and, similar to relative positions in the original transformer model[21], may help the model attend to m/z differences between peaks, which are critical for identification of amino acids in the peptide sequence. The intensity, which is measured with lower precision than the m/z value, is embedded by projection to d dimensions through a linear layer, after which the m/z and intensity embeddings are summed to produce the input peak embedding.

Precursor information, used as input to the decoder, consists of the total mass $m_{\text{prec}} \in R$ and charge state $c_{\text{prec}} \in \{1, ..., 10\}$ associated with the spectrum. We use the same sinusoidal position embedding as peak m/z's for m_{prec}; c_{prec} is embedded using an embedding layer, and the embeddings are summed to obtain the input precursor embedding. Preceding amino acids in the peptide sequence, another decoder input, are also encoded as the sum of an amino acid embedding and a sinusoidal position embedding of their position in the sequence.

Training and inference strategy

Taking the previously described embeddings as input, the transformer outputs scores which are treated as a probability distribution over the amino acid vocabulary for the next position in the sequence at each decoding step. The amino acid vocabulary includes 20 canonical amino acids (with cysteine considered to be carbamidomethylated), post-translationally modified versions of three of them (oxidation of methionine and deamidation of asparagine or glutamine), N-terminal modifications (acetylation, carbamylation, loss of ammonia, and the combination of loss of ammonia and carbamylation), plus a special `stop` token to signal the end of decoding, yielding a total of 28 tokens. During training, the decoder is fed the amino acid prefix for the ground truth peptide following the teacher forcing paradigm[37]. Cross-entropy between the model output probabilities and a binary matrix representing the amino acid sequence of the ground truth peptide is minimized as the objective function. During inference, beam search is used to find the highest-scoring predicted peptide sequence, with k a user-specified value for the number of beams. At each prediction step, for every peptide prefix considered, the k top-scoring amino acids are selected, after which the k top-ranked amino acid sequences are used for the subsequent decoding step. Beams are terminated when the `stop` token is predicted, the predicted peptide mass is similar to (given the precursor mass tolerance) or exceeds the precursor mass, or the pre-defined maximum peptide length of $\ell = 100$ amino acids is reached. As final prediction, the top-scoring peptide sequence that fits the precursor mass tolerance (optionally accounting for isotope offsets) is selected. If no peptide prediction fits the precursor mass tolerance, the top-scoring peptide sequence with a non-matching peptide mass is selected.

Model and training hyperparameters

We train models with nine layers, embedding size $d = 512$, and eight attention heads, yielding a total of ∼47M model parameters. A batch size of 32 spectra and 10^{-5} weight decay is used during training, with a peak learning rate of 5×10^{-4}. The learning rate is linearly increased from zero to its peak value in 100,000 warm-up steps, followed by a cosine shaped decay. The MassIVE-KB model was trained for a single epoch on 30 million PSMs from the MassIVE-KB dataset, while evaluating the performance on the validation set after every 50,000 iterations. The final model weights were taken from the snapshot with the lowest validation loss. These model hyperparameters—number of layers, embedding size, number of attention heads, and learning rate schedule—are used for all downstream experiments unless otherwise specified.

Precursor *m/z* filtering

A critical constraint in *de novo* peptide sequencing requires the difference between the total mass of the predicted peptide m_{pred} and the observed precursor mass m_{prec} of the spectrum to be smaller than a threshold value ϵ (specified in ppm) for the predicted sequence to be plausible: $\Delta m_{ppm} = \frac{|m_{\text{prec}} - m_{\text{pred}}| \times 10^6}{m_{\text{prec}}} < \epsilon$. Therefore, in addition to providing precursor information as an input for the model to learn from, we filter out peptide predictions that do not satisfy the above constraint. The threshold value ϵ is a property of the mass spectrometer that the data is collected with, and hence is known at inference time. Accordingly, we choose ϵ based on the precursor mass error tolerance used in the database search to obtain ground truth peptide sequences for the test data.

Code availability

The transformer model was implemented using PyTorch[38] and PyTorch Lightning[39]. Additionally, NumPy (`https://doi.org/10.1038/s41586-020-2649-2`), Pandas (`10.25080/Majora-92bf1922-00a`), and Scikit-Learn (`https://jmlr.csail.mit.edu/papers/v12/pedregosa11a.html`) were used for scientific data processing; and spectrum_utils[40], Pyteomics[38], and ppx[41] were used to process MS/MS data. Matplotlib[42] and Seaborn (REF: https://doi.org/10.21105/joss.03021) were used for visualization purposes.

Casanovo's source code and trained model weights are available under the Apache 2.0 license at `https://github.com/Noble-Lab/casanovo`. Casanovo$_{bm}$ is trained using version 2.1.1, whereas Casanovo and Casanovo$_{ne}$ use version 3.2.0.

Datasets

MassIVE-KB dataset

A large-scale, heterogeneous dataset derived from the MassIVE knowledge base (MassIVE-KB; v.2018-06-15) was used to develop Casanovo[23]. The MassIVE-KB data set consists of 31 TB of human data from 227 public proteomics datasets, containing over 669 million MS/MS spectra. MassIVE-KB contains a designated subset of 30,506,973 "high quality" PSMs, identified by applying a strict (∼0%) PSM-level FDR filter and then selecting at most 100 PSMs for each combination of peptide sequence and charge. These 30 million PSMs were randomly split so that the training, validation and test sets are disjoint at the peptide-level and consist of approximately 28 million training PSMs, 1 million validation PSMs, and 1 million test PSMs.

Nine-species benchmark dataset

We created a new version of the nine-species benchmark originally described by Tran *et al.*[8] To do so, we downloaded the RAW files from the same nine PRIDE projects (Supplementary Table S2) and converted them to MGF format using the ThermoRawFileParser v1.3.4. We also downloaded the corresponding nine Uniprot reference proteomes and constructed a Tide index for each one, using Crux version 4.1. For one species (*Vigna mungo*), no reference proteome is available, so we used the proteome of the closely related species *Vigna radiata*. We specified Cys carbamidomethylation as a static modification and allowed for the following variable modifications: Met oxidation, Asn deamidation, Gln deamidation, N-term acetylation, N-term carbamylation, N-term NH_3 loss, and the combination of N-term carbamylation and NH_3 loss by using the tide-index options `--mods-spec 1M+15.994915,1N+0.984016,1Q+0.984016 --nterm-peptide-mods-spec 1X+42.010565,1X+43.005814,1X-17.026549,1X+25.980265 --max-mods 3`. Note that one of the nine experiments (*Mus musculus*) was performed using SILAC labeling, but we searched without SILAC modifications and hence include in the benchmark only PSMs from unlabeled peptides. Each index also contains a shuffled decoy peptide corresponding to each target peptide. Each MGF file was searched against the corresponding index using the precursor window size and fragment bin tolerance specified in the original study (Supplementary Table S2). We used XCorr scoring with Tailor calibration[43], and we allowed for 1 isotope error in the selection of candidate peptides. All search results were then analyzed jointly per species using the Crux implementation of Percolator, with default parameters. For the benchmark, we retained all PSMs with Percolator q value < 0.01. We identified 13 MGF files with fewer than 100 accepted PSMs, and we eliminated all of these PSMs from the benchmark. We then post-processed the PSMs to eliminate peptides that are shared between species. Among the 229,984 unique peptides, we identified 3797 (1.7%) that occur in more than one species. For each such peptide, we selected one of the associated species at random and then eliminated all PSMs containing that peptide in other species. The final benchmark dataset consists of 2.8 million PSMs drawn from 343 RAW files. The revised nine-species benchmark is available on MassIVE at `https://doi.org/doi:10.25345/C52V2CK8J`.

Evaluation metrics

We use precision calculated at the amino acid and peptide levels[8,11,13] as a function of coverage over the test set as performance measures to evaluate the quality of a given model's predictions. In each case, for each spectrum we compare the predicted sequence to the ground truth peptide from the database search. Following Tran *et al.*[8], for the amino acid-level measures we first calculate the number N^a_{match} of matched amino acid predictions, defined as all predicted amino acids which (1) differ by <0.1 Da in mass from the corresponding ground truth amino acid, and (2) have either a prefix or suffix that differs by no more than 0.5 Da in mass from the corresponding amino acid sequence in the ground truth peptide. We then define amino acid-level precision as $N^a_{\text{match}}/N^a_{\text{pred}}$, where N^a_{pred} is the number of predicted amino acids. For peptide predictions, a predicted peptide is considered a correct match if all of its amino acids are matched. Among a collection of N^p_{orig} spectra, if our model makes predictions on a subset of N^p_{pred} and correctly predicts N^p_{match} peptides, we define coverage as $N^p_{\text{pred}}/N^p_{\text{orig}}$ and peptide-level precision as $N^p_{\text{match}}/N^p_{\text{pred}}$. To plot a precision-coverage curve, we sort predictions by the confidence score provided by the model. Amino acid-level confidence scores are obtained by applying a softmax to the output of the transformer decoder, which is a proxy for the probability of each predicted amino acid to occur in the given position along the peptide

sequence. Casanovo directly outputs amino acid-level confidence scores, and we use the mean score over all amino acids as a peptide-level confidence score.

Competing methods

We downloaded DeepNovo weights from `https://github.com/nh2tran/DeepNovo/tree/PNAS` on Sep 6, 2022. Similar to Casanovo$_{bm}$, DeepNovo was trained in a cross-validated fashion using the original nine-species benchmark, systematically training on eight species and testing on the remaining species. Accordingly, nine different sets of pre-trained DeepNovo weights were available, and the corresponding set of weights were used for testing on each species data set.

We downloaded Novor v1.05.0573 from `https://github.com/compomics/searchgui/tree/master/resources/Novor` on Dec 3, 2022.

Creating a non-enzymatic dataset

To create a dataset of PSMs that does not exhibit tryptic bias, we selected PSMs with a uniform distribution of amino acids at the C-terminal peptide positions from two datasets: MassIVE-KB[23] and PROSPECT[27]. The MassIVE-KB dataset contains 30 million PSMs and consists entirely of data generated using trypsin; hence, only a small proportion of the MassIVE-KB peptides do not end in K or R, corresponding to those that appear at the C-terminus of the corresponding protein. The PROSPECT dataset is a collection of 61 million PSMs generated from synthetic peptides. To avoid overrepresentation of some peptides in this dataset, we randomly selected at most 100 PSMs per unique peptide, similar to the processing that was done during the creation of the MassIVE-KB dataset. This pre-selection step reduced the size of the PROSPECT dataset to 44 million PSMs. Finally, to create a non-enzymatic dataset containing 1 million peptides, we selected 50,000 PSMs for each canonical amino acid. These PSMs were selected at random from MassIVE-KB, then supplemented as necessary from PROSPECT to obtain the desired count (Supplementary Table S1). This dataset contained PSMs from 282,227 unique peptides, which were randomly split into training, validation and test sets in the ratio 80/10/10. The Casanovo model was fine-tuned on the training dataset, selecting the model with minimal validation loss, which occurred after 9 epochs.

Immunopeptidome analysis

For the analysis of the MHC class I peptides, we used the Tide search engine and adopted search settings from the original publication[5]. To create the peptide index, we ran tide-index allowing M oxidation or phosphorylation of S/T/Y, with a maximum of one modification per peptide. We set the digestion to be "non-specific-digest," allowed zero missed cleavages, and specified a peptide length range of 8–15 amino acids. Using the canonical human reference proteome downloaded from Uniprot on July 17, 2022, the resulting index contains 286,319,284 peptides and an equal number of shuffled decoy peptides. We searched the data using the tide-index command, specifying a precursor window size of 30 ppm and using Tailor calibration. The resulting sets of PSMs from all five runs were analyzed jointly using Percolator with default settings. All of the above commands were implemented within Crux[44] version 4.1-2fab3c91-2022-11-09. For comparative analysis between Casanovo predictions and database search results, only sequences within a peptide length range of 9–15 amino acids were considered. NetMHCpan-4.1 was used to predict MHC binding affinities for peptide sequences[30]. Binding affinity was predicted for 9-mer amino acid motifs in reference to the HLA-A02:01, HLA-A02:17, HLA-B41:01, HLA-B40:02, HLA-C02:02, and HLA-C17:01 alleles of the MHC molecule.

Metaproteomics analysis

We analyzed data from six mass spectrometry runs, three replicates each from the Bering Strait (BSt) and the Chukchi Sea (CS)[31], downloaded in mzXML format from `https://noble.gs.washington.edu/proj/metapeptide`. From the same URL, we also downloaded the two corresponding metapeptide databases, and we downloaded the environmental non-redundant database (env_nr) from NCBI on Nov 12, 2022. We used Tide[28] to build three peptide indices from the two metapeptide databases and from env_nr with default

parameters, except we allowed three methionine oxidations per peptide and up to 1 missed cleavage. The resulting indices contained 41,665,963 peptides (CS), 34,116,884 peptides (BSt), and 310,021,565 peptides (env_nr), respectively, as well as an equal number of shuffled decoy peptides. Each mzXML file was searched against the relevant metapeptide database and against env_nr using the tide-search command, specifiying a precursor window of 10 ppm, allowing one isotope error, and using Tailor calibration. Finally, we used Percolator[29] with default parameters to jointly analyze the search results from each set of three runs against the same peptide index. All of the above commands were implemented within Crux[44] version 4.1-2fab3c91-2022-11-09.

To estimate the error rate for the matching of Casanovo predicted peptides against a protein database, we developed a procedure akin to the target-decoy competition used in false discovery rate estimation for mass spectrometry database search[45]. To do so, we created a decoy protein database by randomly shuffling each protein sequence. We then asked whether each Casanovo prediction appears in the target (i.e., unshuffled) or decoy protein list, marking each predicted peptide as matching to the target, decoy, or neither. Peptides assigned to both were randomly assigned to either the target or decoy. We then segregated the peptides by length, and for each length we sorted the Casanovo predictions by confidence score. At each position k in the ranked list, we estimated the rate of random matching among the target matches as D_k/T_k, where D_k (respectively, T_k) is the number of decoys (respectively, targets) with rank smaller than k. We then selected the largest value of k such that $D_k/T_k < \alpha$, for some specified random matching rate α. In this work, we used $\alpha = 0.01$.

We used the online version of Prosit[32] at `https://www.proteomicsdb.org/prosit/` to predict peak intensities and retention times using the Prosit_2020_intensity_hcd and Prosit_2019_irt models, respectively. A fixed collision energy of 27 was used for all peptides based on metadata from the spectrum files. Spectrum peaks predicted by Prosit were matched to observed peaks using a 0.05 Da fragment m/z tolerance to calculate the cosine similarity between the predicted and experimental spectra.

Acknowledgements

This work is in part supported by CCF-2019844 as a part of the NSF Institute for Foundations of Machine Learning (IFML).

References

1. Aebersold, R. & Mann, M. Mass-spectrometric exploration of proteome structure and function. *Nature* **537,** 347–355 (2016).
2. Eng, J. K., McCormack, A. L. & Yates, III, J. R. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *Journal of the American Society for Mass Spectrometry* **5,** 976–989 (1994).
3. Eng, J. K., Searle, B. C., Clauser, K. R. & Tabb, D. L. A face in the crowd: recognizing peptides through database search. *Molecular and Cellular Proteomics* **10** (2011).
4. Bittremieux, W. *et al.* Quality control in mass spectrometry-based proteomics. *Mass Spectrometry Reviews* **37,** 697–711 (2018).
5. Stopfer, L. E., Mesfin, J. M., Joughin, B. A., Lauffenburger, D. A. & White, F. M. Multiplexed relative and absolute quantitative immunopeptidomics reveals MHC I repertoire alterations induced by CDK4/6 inhibition. *Nature Communications* **11,** 1–14 (2020).
6. Mayer, R. L. & Impens, F. Immunopeptidomics for next-generation bacterial vaccine development. *Trends in Microbiology* **29,** 1034–1045 (2021).
7. Hunt, D. F. *et al.* Characterization of Peptides Bound to the Class I MHC Molecule HLA-A2.1 by Mass Spectrometry. *Science (New York, N.Y.)* **255,** 1261–1263. ISSN: 0036-8075 (Mar. 1992).
8. Tran, N. H., Zhang, X., Xin, L., Shan, B. & Li, M. De novo peptide sequencing by deep learning. *Proceedings of the National Academy of Sciences of the United States of America* **31,** 8247–8252 (2017).

9. Muth, T., Benndorf, D., Reichl, U., Rapp, E. & Martens, L. Searching for a needle in a stack of needles: challenges in metaproteomics data analysis. *Molecular Biosystems* **9,** 578–585 (2013).

10. Taylor, J. A. & Johnson, R. S. Sequence database searches via *de novo* peptide sequencing by tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **11,** 1067–1075 (1997).

11. Ma, B. *et al.* PEAKS: powerful software for peptide *de novo* sequencing by tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **17,** 2337–2342 (2003).

12. Dancik, V., Addona, T., Clauser, K., Vath, J. & Pevzner, P. *De novo* peptide sequencing via tandem mass spectrometry. *Journal of Computational Biology* **6,** 327–342 (1999).

13. Frank, A. & Pevzner, P. Pepnovo: de novo peptide sequencing via probabilistic network modeling. *Analytical Chemistry* **77,** 964–973 (2005).

14. Fischer, B. *et al.* A hidden Markov model for de novo peptide sequencing. *Advances in Neural Information Processing Systems* **17,** 457–464 (2005).

15. Ma, B. Novor: Real-Time Peptide de Novo Sequencing Software. *Journal of the American Society for Mass Spectrometry* **26,** 1885–1894 (2015).

16. Karunratanakul, K., Tang, H.-Y., Speicher, D. W., Chuangsuwanich, E. & Sriswasdi, S. Uncovering Thousands of New Peptides with Sequence-Mask-Search Hybrid De Novo Peptide Sequencing Framework. *Molecular and Cellular Proteomics* **18,** 2478–2491 (2019).

17. Qiao, R. *et al.* Computationally instrument-resolution-independent de novo peptide sequencing for high-resolution devices. *Nature Machine Intelligence* **3,** 420–425 (2021).

18. Qi, C. R., Su, H., Mo, K. & Guibas, L. J. *PointNet: deep learning on point sets for 3D classification and segmentation* in *Proceedings of the IEEE Conference On Computer Vision and Pattern Recognition* (2016), 652–660.

19. Yang, H., Chi, H., Zeng, W., Zhou, W. & He, S. pNovo 3: precise de novo peptide sequencing using a learning-to-rank framework. *Bioinformatics* **35,** i83–i90 (2019).

20. Zhou, X. *et al.* pDeep: predicting MS/MS spectra of peptides with deep learning. *Analytical Chemistry* **89,** 12690–12697 (2017).

21. Vaswani, A. *et al.* Attention Is All You Need. en. *Advances in Neural Information Processing Systems* **30** (2017).

22. Yilmaz, M., Fondrie, W. E., Bittremieux, W., Oh, S. & Noble, W. S. De novo *mass spectrometry peptide sequencing with a transformer model* in *Proceedings of the International Conference on Machine Learning* (2022), 25514–25522.

23. Wang, M. *et al.* Assembling the Community-Scale Discoverable Human Proteome. *Cell Systems* **7,** 412–421.e5 (4 2018).

24. Rives, A. *et al.* Biological structure and function emerge from scaling unsupervised learning to 250 million protein sequences. *Proceedings of the National Academy of Sciences of the United States of America* **118,** e2016239118 (2021).

25. Avsec, Z. *et al.* Effective gene expression prediction from sequence by integrating long-range interactions. *Nature Methods* **18,** 1196–1203 (2021).

26. Bittremieux, W., May, D. H., Bilmes, J. & Noble, W. S. A learned embedding for efficient joint analysis of millions of mass spectra. *Nature Methods* **19,** 675–678 (2022).

27. Shouman, O., Gabriel, W., Giurcoiu, V.-G., Sternlicht, V. & Wilhelm, M. *PROSPECT: Labeled Tandem Mass Spectrometry Dataset for Machine Learning in Proteomics* in *Thirty-sixth Conference on Neural Information Processing Systems Datasets and Benchmarks Track* (2022).

28. Diament, B. & Noble, W. S. Faster SEQUEST searching for peptide identification from tandem mass spectra. *Journal of Proteome Research* **10,** 3871–3879 (2011).

29. Käll, L., Canterbury, J., Weston, J., Noble, W. S. & MacCoss, M. J. A semi-supervised machine learning technique for peptide identification from shotgun proteomics datasets. *Nature Methods* **4,** 923–25 (2007).

30. Reynisson, B., Alvarez, B., Paul, S., Peters, B. & Nielsen, M. NetMHCpan-4.1 and NetMHCIIpan-4.0: improved predictions of MHC antigen presentation by concurrent motif deconvolution and integration of MS MHC eluted ligand data. *Nucleic Acids Research* **48,** W449–W454 (2020).

31. May, D. H. *et al.* An Alignment-Free "Metapeptide" Strategy for Metaproteomic Characterization of Microbiome Samples Using Shotgun Metagenomic Sequencing. *Journal of Proteome Research* **15,** 2697–2705 (2016).

32. Gessulat, S. *et al.* Prosit: proteome-wide prediction of peptide tandem mass spectra by deep learning. *Nature Methods* **16,** 509 (2019).

33. Henikoff, S. & Henikoff, J. G. Amino acid substitution matrices from protein blocks. *Proceedings of the National Academy of Sciences of the United States of America* **89,** 10915–10919 (1992).

34. Johnson, R. S. *et al.* Assessing protein sequence database suitability using de novo sequencing. *Molecular & Cellular Proteomics* **19,** 198–208 (2020).

35. Kertesz-Farkas, A., Keich, U. & Noble, W. S. Tandem mass spectrum identification via cascaded search. *Journal of Proteome Research* **14,** 3027–3038 (2015).

36. Beslic, D., Tscheuschner, G., Renard, B. Y., Weller, M. G. & Muth, T. Comprehensive evaluation of peptide de novo sequencing tools for monoclonal antibody assembly. *Briefings in Bioinoformatics.* Advance online access (Dec. 2022).

37. Williams, R. J. & Zipser, D. A learning algorithm for continually running fully recurrent neural networks. *Neural Computation* **1,** 270–280 (1989).

38. Paszke, A. *et al.* in *Advances in Neural Information Processing Systems 32* 8024–8035 (Curran Associates, Inc., Vancouver, Canada, 2019). `http://papers.neurips.cc/paper/9015-pytorch-an-imperative-style-high-performance-deep-learning-library.pdf`.

39. Falcon, W. & Team, T. *PyTorch Lightning The lightweight PyTorch wrapper for high-performance AI research. Scale your models, not the boilerplate* 2019.

40. Bittremieux, W. spectrum_utils: A Python Package for Mass Spectrometry Data Processing and Visualization. *Analytical Chemistry* **92,** 659–661 (2020).

41. Fondrie, W., Bittremieux, W. & Noble, W. S. ppx: Programmatic access to proteomics data repositories. *Journal of Proteome Research* **20.** PMC8457024, 4621–4624 (2021).

42. Hunter, J. D. Matplotlib: A 2D graphics environment. *Computing in science & engineering* **9,** 90–95 (2007).

43. Sulimov, P. & Kertész-Farkas, A. Tailor: A Nonparametric and Rapid Score Calibration Method for Database Search-Based Peptide Identification in Shotgun Proteomics. *Journal of Proteome Research* **19,** 1481–1490 (2020).

44. Park, C. Y., Klammer, A. A., Käll, L., MacCoss, M. P. & Noble, W. S. Rapid and accurate peptide identification from tandem mass spectra. *Journal of Proteome Research* **7,** 3022–3027 (2008).

45. Elias, J. E. & Gygi, S. P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nature Methods* **4,** 207–214 (2007).

Supplementary information

	MassIVE-KB count	MassIVE-KB selected	PROSPECT count	PROSPECT selected
A	24910	24910	859335	25090
C	11473	11473	103935	38527
D	44654	44654	358715	5346
E	42977	42977	745564	7023
F	38183	38183	1273556	11817
G	21640	21640	536491	28360
H	166398	50000	433266	0
I	12984	12984	646996	37016
K	16289160	50000	14157777	0
L	50002	50000	3235902	0
M	19405	19405	537652	30595
N	40867	40867	356285	9133
P	9685	9685	211181	40315
Q	33572	33572	595464	16428
R	13589301	50000	15668902	0
S	28389	28389	608722	21611
T	15845	15845	544457	34155
V	26011	26011	1172497	23989
W	4261	4261	431324	45739
Y	35180	35180	1790980	14820
Total	30504897	610036	44269001	389964

Table S1: **Creating a non-enzymatic dataset by sampling from PROSPECT and MassIVE-KB.** PROSPECT was first downsampled to include at most 100 PSMs per peptide sequence. MassIVE-KB and PROSPECT were then segregated by C-terminal amino acid, and we randomly selected from each category from MassIVE-KB, supplementing as necessary from PROSPECT to obtain 50,000 PSMs per terminal amino acid.

PRIDE	Species	Uniprot	Files	Spectra	PSMs	precursor	fragment
PXD005025	*Vigna mungo*	UP000087766	24	932848	107429	20	0.05
PXD004948	*Mus musculus*	UP000000589	13	306786	23984	10	0.05
PXD004325	*Methanosarcina mazei*	UP000033058	72	3728183	263595	10	0.05
PXD004565	*Bacillus subtilis*	UP000001570	106	4336428	1344611	30	0.05
PXD004536	*Candidatus endoloripes*	UP000094849	11	2272023	80615	20	0.05
PXD004947	*Solanum lycopersicum*	UP000004994	60	603506	180022	15	0.05
PXD003868	*Saccharomyces-cerevisiae*	UP000002311	27	1477397	571655	20	0.05
PXD004467	*Apis mellifera*	UP000005203	17	823169	197102	20	0.05
PXD004424	*H. sapiens*	UP000005640	26	684821	43181	20	0.02
Total			343	15,165,161	2,812,194		

Table S2: **The nine-species benchmark.** The final two columns specify the precursor window size (in ppm) and fragment bin size (in Da) used in the database search step. No reference proteome is available for *Vigna mungo*, so the proteome for the closely related species *Vigna radiata* was used instead.

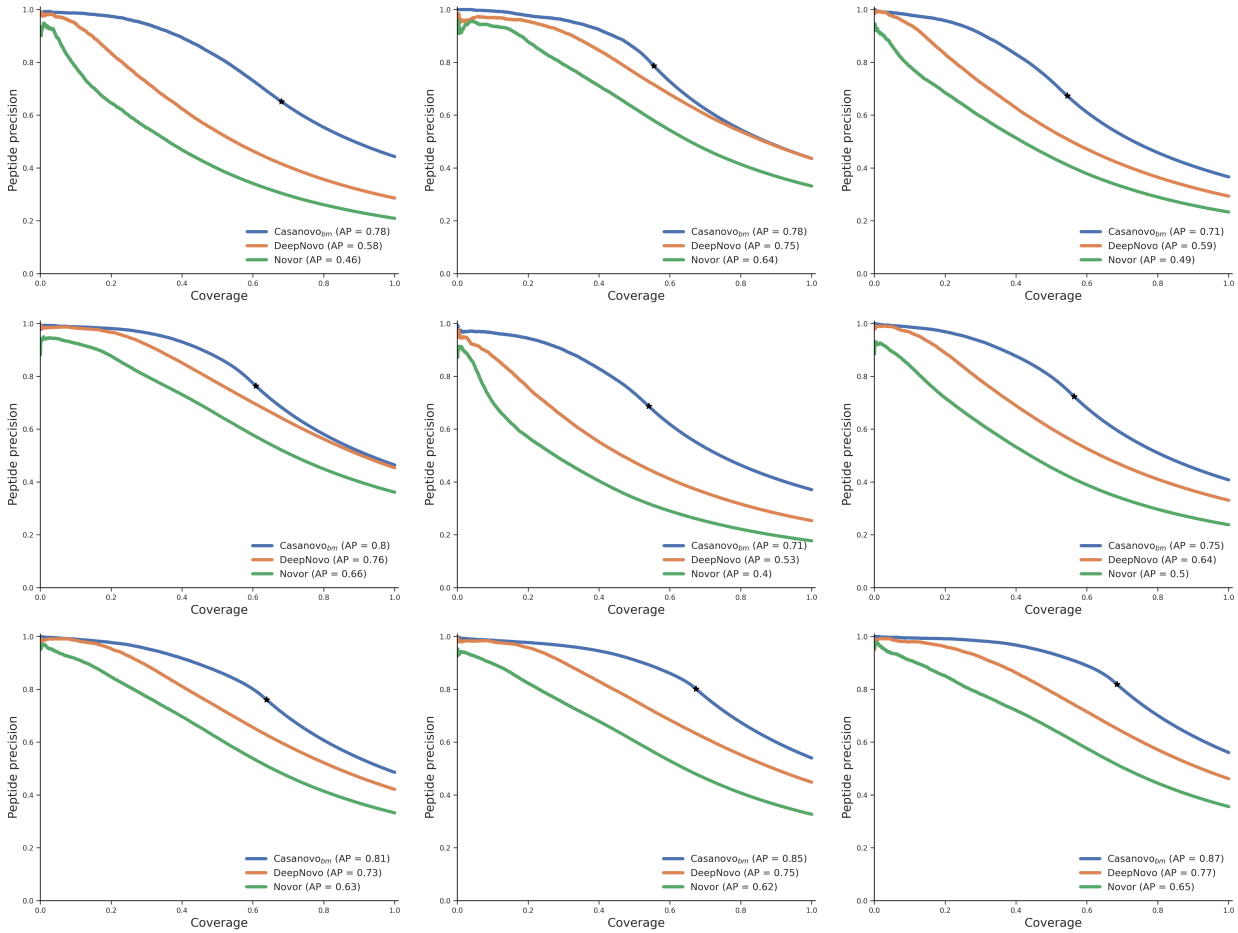


Figure S1: **Casanovo$_{bm}$ outperforms Novor and DeepNovo on the original nine species benchmark.** Each panel evaluates the peptide-level performance on the held-out species in the nine species benchmark. For Casanovo$_{bm}$ all peptides that pass the precursor m/z filter are ranked above peptides that do not pass the filter, and the boundary is indicated by a star on the curve.

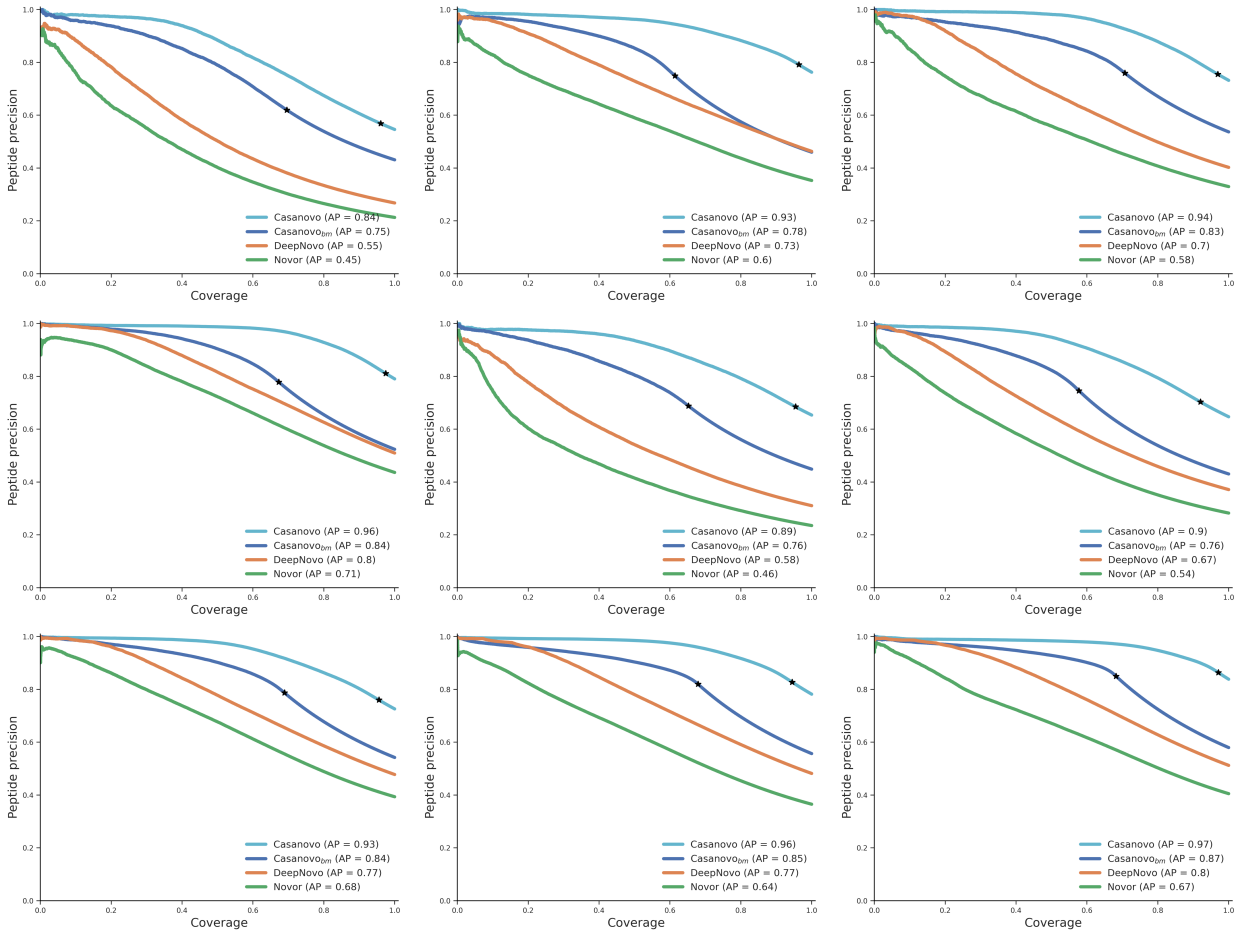
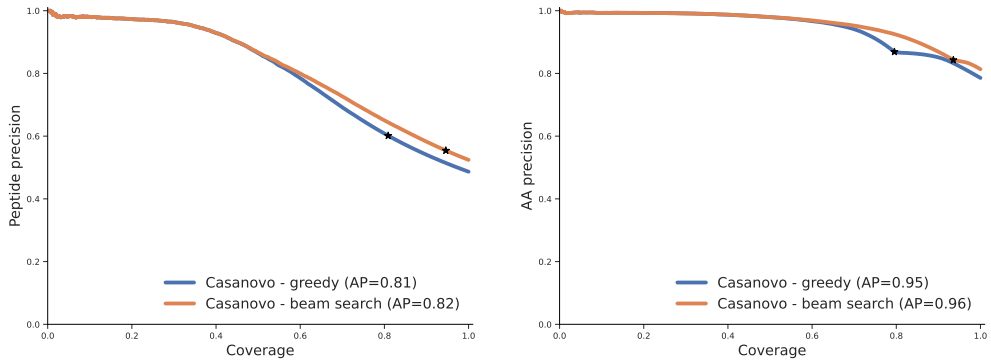


Figure S2: **Casanovo outperforms Novor, DeepNovo, and Casanovo$_{bm}$ on the revised nine-species benchmark** Each panel evaluates the peptide-level performance on the held-out species in the nine species benchmark. For Casanovo and Casanovo$_{bm}$ all peptides that pass the precursor m/z filter are ranked above peptides that do not pass the filter, and the boundary is indicated by a star on each curve.



A B

Figure S3: **Comparison of greedy and beam-search decoding.** (A) The plot shows precision-coverage curves for the same Casanovo model for which either greedy decoding or beam-search decoding with ten beams was used as the peptide decoding strategy. Mouse data set from the revised benchmark was used for this analysis. (B) Similar to panel (A), but showing amino acid-level precision and coverage for the same data set.

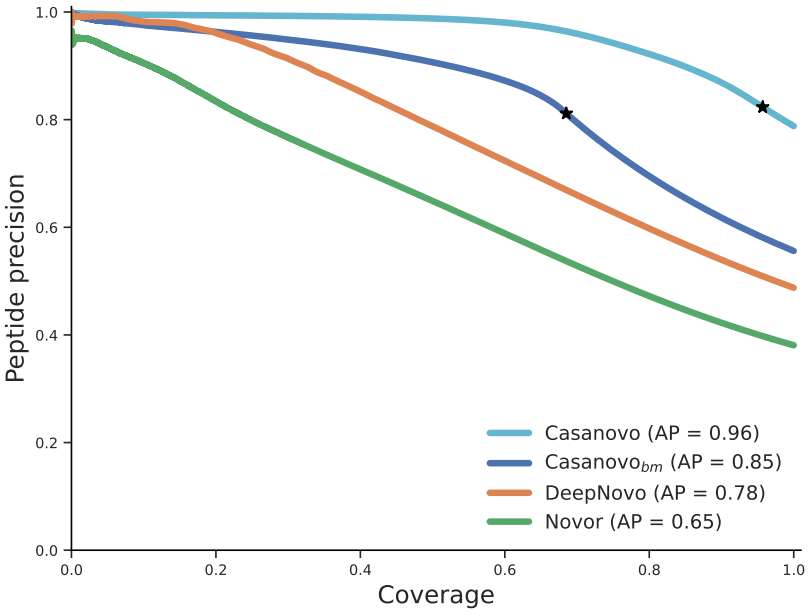


Figure S4: **Casanovo outperforms Novor, DeepNovo, and Casanovo$_{bm}$ on the revised nine-species benchmark, even when PTMs unavailable to DeepNovo or Novor are eliminated.** The figure plots peptide-level precision as a function of coverage for all species in the nine-species benchmark for Casanovo, DeepNovo and Novor. Casanovo maintains higher peptide-level precision across the full coverage range over Novor, DeepNovo, and Casanovo$_{bm}$ on the aggregated, revised nine-species benchmark. MS/MS spectra associated with modifications that cannot be detected by DeepNovo and Novor are eliminated.

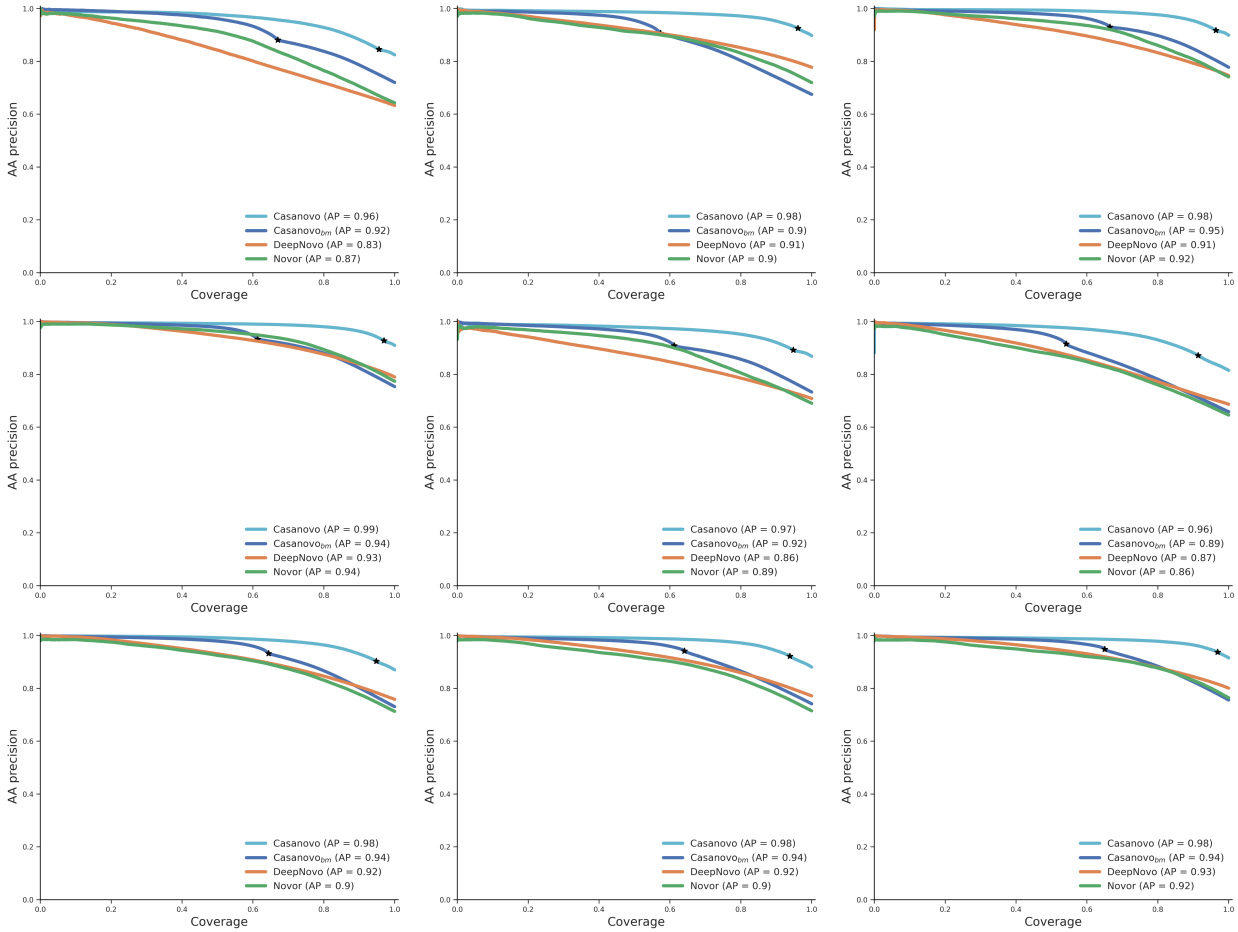


Figure S5: **Casanovo outperforms Novor, DeepNovo, and Casanovo$_{bm}$ at the amino acid-level on the nine-species benchmark** Each panel evaluates the amino acid-level performance on the held-out species in the nine species benchmark. For Casanovo and Casanovo$_{bm}$ all peptides that pass the precursor m/z filter are ranked above peptides that do not pass the filter, and the boundary is indicated by a star on each curve.

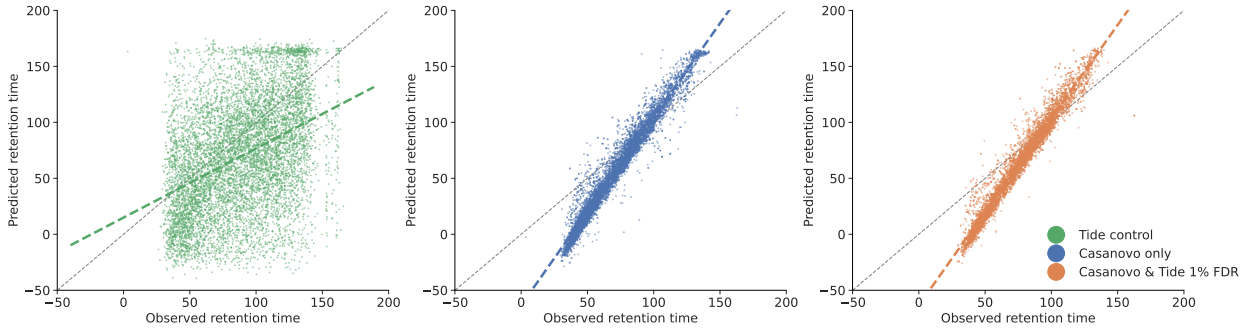


Figure S6: **Confident Casanovo assignments have retention times that are highly correlated with Prosit predictions.** We compared the observed retention times against Prosit-predicted retention times for three groups of peptides: (1) peptides predicted only by Casanovo that match to the relevant database at 1% error rate, (2) peptides identified by both Casanovo and database search with a 1% FDR threshold, and (3) peptides identified by database search with FDR >10%. The dashed line in each plot represents the ordinary least squares linear regression.

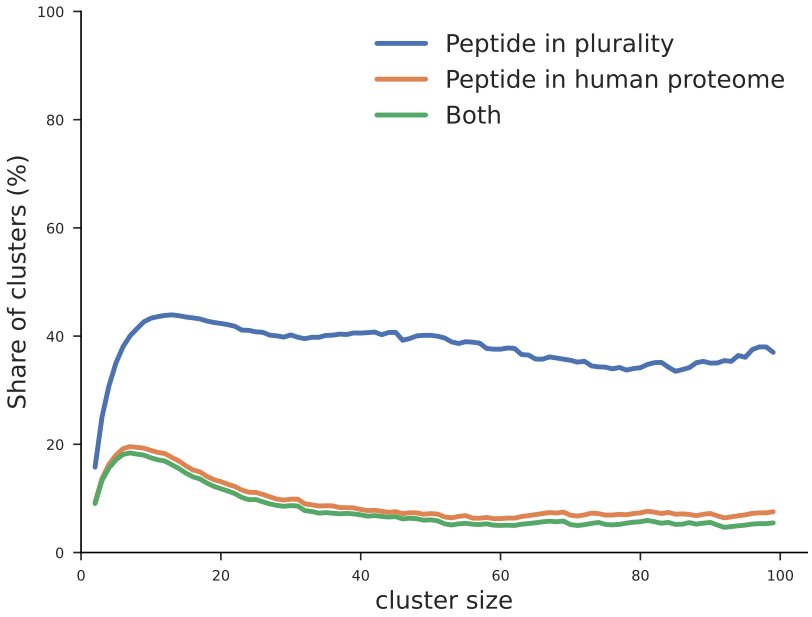


Figure S7: **Casanovo assigns new peptides to dark matter clusters of various sizes.** We evaluated the fraction of clusters which have a Casanovo peptide in plurality, have a unique Casanovo peptide matching to human proteome or satisfy both criteria among all previously unidentified clusters larger than a given size.

In human proteome	In Casanovo peptide	BLOSUM score	Count
L	V	1	743
V	L	1	669
E	Q	2	492
N	D	1	426
R	K	2	368
E	H	0	366
E	D	2	340
A	S	1	315
M	H	-2	314
L	F	0	314

Table S3: **The most common amino acid swaps have positive BLOSUM scores.** We found that the top ten most common single amino acid substitutions that can be explanied with a single nucleotide polymorphism detected by Casanovo are enriched for positive BLOSUM scores.