A modular, immunopeptidogenomic (iPepGen) analysis pipeline for discovery, verification, and prioritization of cancer peptide neoantigen candidates

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Abstract

Characterizing tumor-specific neoantigen peptides, derived from genomic or transcriptomic aberrations and presented to the immune system, is critical for immuno-oncology studies. To this end, the modular iPepGen immunopeptidogenomics pipeline provides these functions: (1) Neoantigen prediction and protein database generation from genomic or transcriptomic sequencing data; (2) Peptide identification (3) Verification from immunopeptidomic mass spectral data; (4) Neoantigen classification and visualization; (5) Candidate prioritization for further study. Easy access via a publicly available, scalable cloud-based gateway coupled with online, interactive training materials streamlines the adoption by cancer researchers who require immunopeptidogenomic analysis tools but lack advanced computational expertise and resources.

Keywords

Neoantigen, immunopeptidomics, immuno-oncology, mass spectrometry, next-generation sequencing, multi-omics, Galaxy, bioinformatics, workflow

Background

Neoantigen peptides, derived from non-reference proteoforms uniquely expressed in tumor cells, are an increasingly important class of targets in cancer immunotherapy. These proteoforms may arise from a variety of genomic, transcriptomic, and post-translational mechanisms, including non-synonymous mutations, gene fusions [1], aberrant splicing, de novo transcription from non-coding regions [2], derepression of transposable elements, post-translational modification (PTM) [3], and translation of viral open reading frames in virus-associated cancers [4]. These alterations yield tumor-specific antigens absent from normal tissues and thus bypass central tolerance [5–8]. In addition to neoantigens, tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs) derived from reference proteoforms whose expression is either enriched (TAAs) or only found in cancerous cells (TSAs) are of interest for immunotherapy development [9].

Once synthesized, aberrant proteoforms are degraded by cellular proteolytic machinery. In the cytosol, peptides of 8–11 amino acids are generated and presented by Major Histocompatibility complex (MHC) class I (called Human Leukocyte Antigen complex, HLA-A, -B, -C in humans) [10]. Meanwhile, longer peptides from extracellular proteins (10–25 amino acids) are presented via MHC class II (HLA-D family in humans) [11]. These complexes are displayed on the cell surface and recognized by T cells. It has been estimated that up to 10% of these peptides may elicit immunogenic responses when derived from non-reference sequences [12]. Despite this, tumors

can escape immune surveillance via multiple mechanisms [13]. However, when MHC-bound neoantigen peptides are successfully identified, they can be leveraged for immunotherapy, including adoptive T cell transfer, immune checkpoint blockade, and prophylactic vaccination [14–18].

Identifying and predicting neoantigen candidates from genomic and transcriptomic data. The ability to identify neoantigen candidates from genomic and transcriptomic sequences has advanced rapidly due to improvements in next-generation sequencing (NGS) and customized computational pipelines [8,19]. Numerous tools have been developed for identifying potential neoantigens encoded by tumor-specific alterations [20–24]. These tools typically utilize RNA-Seq data or whole-exome sequencing to genotype alleles coding for MHC protein subunits and predict binding affinities of potential neoantigen peptides to the MHC based on biochemical and structural modeling [25–28]. Higher predicted binding affinity to specific MHC (or HLA) alleles is an indicator of immune recognition and the possibility of a peptide to elicit an immunogenic response. Deep learning tools have also emerged that seek to predict potential immunogenicity for neoantigen sequences identified by genome or transcriptome sequencing [29–31].

Beyond prediction: confirming MHC-bound neoantigens via mass spectrometry (MS)-based immunopeptidomics. Although important, NGS-based approaches ultimately provide only a predictive view of antigen presentation, which presents limitations [8,32]. These include the inability to confirm proteoform translation, peptide processing, or MHC presentation. Predictions also fail to capture PTMs, which are known to modulate immunogenicity [33], are prone to prediction bias [34,35], and are often missed as antigenic peptides [36]. Empirical follow-up studies have shown that only a small fraction of predicted neoantigens from NGS data are processed, bound to the MHC, and truly immunogenic [17]. These constraints have motivated the development of mass spectrometry (MS)-based immunopeptidomics approaches that allow direct identification of peptides presented by MHC complexes [37–39].

In immunopeptidomics, the intact MHC and bound peptides are affinity-enriched and analyzed by liquid chromatography (LC) tandem mass spectrometry (MS/MS). Tandem mass spectra are then matched to custom protein databases that include both canonical reference sequences and tumor-specific neoantigen candidates inferred from NGS data. This strategy allows the detection of neoantigens and TAAs/TSAs in an unbiased, high-throughput manner, including peptides with PTMs that might influence immunogenicity [33]. Originally developed by Hunt and colleagues [40], MS-based immunopeptidomics has matured and now enables the routine detection of thousands of MHC-bound peptides from cellular and tissue sources [39,41], significantly expanding the scope of immuno-oncology research [8,38].

Informatics requirements for neoantigen prediction and confirmation via immunopeptidogenomics. The integration of NGS and MS data for comprehensive neoantigen discovery, appropriately called "immunopeptidogenomics" by Purcell and colleagues [42], poses unique informatics requirements and challenges. These include managing the heterogeneity of NGS alterations for predicting neoantigens and constructing sample-specific databases; efficient and sensitive peptide spectrum match (PSM) generation from MS/MS searched against customized protein sequence databases using non-specific enzyme constraints [33,43,44], and, if desired, analyzing PTMs; quantifying identified peptides via label-free methods [45,46].

Some pipelines have been described that focus on the analysis of MS-based immunopeptidomics data [47–49]. To varying degrees, these bioinformatic platforms offer the ability to identify potential genomic/transcriptomic aberrations from NGS data and utilize tools for predicting MHC binding to help in prioritization. Some of the platforms [47,48] use publicly available, established software such as MaxQuant [50] or commercial algorithms [42] for generating PSMs from MS/MS data; however, none of these algorithms are optimized for analysis of MHC-bound peptides using "no enzyme" settings, rather than peptides generated by trypsin digestion, as is common in proteomics applications. Recently, a sophisticated multi-omics platform called NeoDisc has been described that is openly available for non-commercial use [51]. NeoDisc offers a complete pipeline with modules for neoantigen prediction, peptide identification from immunopeptidomic MS/MS data, and advanced methods, including a deep learning algorithm for prioritizing candidates for further study. However, this platform requires substantial technical expertise for installation, as well as local computational infrastructure to support its complex set of tools and their computing requirements. It also lacks a GUI, limiting its accessibility and usage by non-expert users who may be new to immunopeptidogenomics. Moreover, the addition of new or experimental tools into these previously described pipelines requires significant software developer expertise, beyond the reach of most non-expert users.

The iPepGen pipeline and the Galaxy ecosystem as a unique solution for immunopeptidogenomic informatics. To address current limitations, we have developed the modular immunopeptidogenomic (iPepGen) pipeline for the discovery and characterization of neoantigens. Deployment in the Galaxy ecosystem [52,53] provides a web-based environment and user interface, immediately accessible via cloud-hosted servers [54], that supports reproducible bioinformatics analyses. Tools are openly available via the Galaxy Tool Shed and can be deployed on public cloud servers or institutional high-performance computing (HPC) resources. Galaxy supports comprehensive NGS and MS-based proteomics workflows [55], with built-in provenance tracking, visualizations, and shareable software workflows and analysis histories. The powerful Galaxy Training Network (GTN) [56,57] further promotes community adoption by offering interactive training resources for new users. Leveraging these strengths,

iPepGen integrates validated tools for neoantigen prediction, peptide identification and verification, genomic mapping, and immunogenicity assessment within a unified and accessible framework complete with training resources. Taken together, these features lower the entry bar for adoption by new users, especially those lacking advanced computational expertise and/or access to HPC resources. Overall, the iPepGen pipeline offers a unique informatics resource built to advance studies by a growing community of immuno-oncology researchers using immunopeptidogenomics for neoantigen discovery and characterization.

Results

We implemented the iPepGen pipeline within the Galaxy ecosystem to support end-to-end neoantigen discovery and prioritization by integrative analysis of NGS and LC-MS/MS immunopeptidomics data, along with other specialized tools. To demonstrate the effectiveness and reproducibility of the modular pipeline, we analyzed paired RNA-Seq and HLA class I immunopeptidomics data from a human malignant peripheral nerve sheath tumor (MPNST) cell line, STS-26T. Moreover, to highlight the flexibility and effectiveness of iPepGen in addressing the challenges of immunopeptidogenomics analysis, we show results from analysis of additional multi-omics data available from previously published studies.

A modular, cloud-based pipeline is easily accessed and used via a web-based interface. The iPepGen pipeline is composed of five modular workflows (**Figure 1**), deployed in the Galaxy bioinformatics environment. These workflows integrate software tools selected and optimized to accomplish specific data analysis tasks and generate results compatible with subsequent analysis by downstream modules in the pipeline. The workflows composing each module are freely available on the European Galaxy server, through the links shown below in **Table 1.** A Galaxy workflow contains all software tools for operation on appropriate input data, along with preset, optimized settings. We have made example input files available for each workflow via Galaxy links **(Table 1)**, which can be imported and recognized as appropriate input formats by the corresponding workflow. Once a workflow is run, Galaxy saves the analysis as a history, which contains all input and result files generated by the workflow. The history can be shared, re-run using adjusted settings, and/or archived. Links to completed histories for each iPepGen module are also shown in **Table 1**, using the example MPNST data. Workflows and histories can be imported into any Galaxy account once a user has registered on the server.

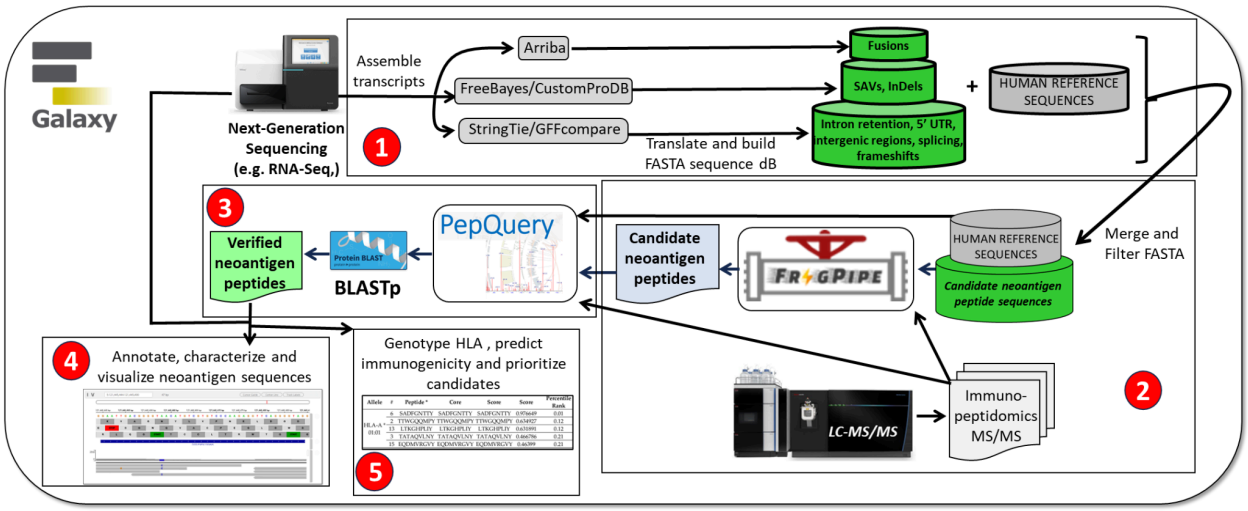

Figure 1. Five modules comprising the Galaxy-based iPepGen pipeline. The modules carry out the following functions: 1) Predict neoantigen candidates from NGS data and generate customized protein sequence databases, including reference and non-reference neoantigen candidate sequences; 2) Discover neoantigen peptide candidates by sequence database searching of tandem mass spectrometry (MS/MS) immunopeptidomics data; 3) Verify discovered peptide candidates through a secondary peptide-centric evaluation against the MS/MS dataset; 4) Visualize and classify the nature of verified neoantigen peptides encoded by the genome and/or transcriptome; 5) Prioritize neoantigens for further exploration and empirical validation.

Table 1. Links for workflows, input data, analysis histories, and training resources for each module of the iPepGen analysis pipeline

Module	Description	Galaxy workflow	Input data for the workflow	Galaxy history	GTN Training resources
1. Predict non-reference neoantigen peptide sequences	Predicts neoantigen peptide sequences encoded by non-reference genes and/or transcripts and generates customized protein sequence databases	***Gene fusion neoantigens*** https://tinyurl.com/ipepgen-gene-fusion-wf	***Gene fusion neoantigens*** https://tinyurl.com/ipepgen-gene-fusion-input	***Gene fusion neoantigens*** https://tinyurl.com/ipepgen-gene-fusion-history	https://tinyurl.com/fusion-gtn
		General non-reference sequence neoantigens https://tinyurl.com/ipepgen-nonref-wf	***General non-reference sequence neoantigens*** https://tinyurl.com/ipepgen-nonref-input	***General non-reference sequence neoantigens*** https://tinyurl.com/ipepgen-nonref-history	https://tinyurl.com/non-reference-gtn
2. Discover candidate neoantigen peptide sequences and tumor-associated antigens	Merges customized and reference databases and uses the FragPipe tool suite to discover PSMs from LC-MS/MS immunopeptidomics data	https://tinyurl.com/ipepgen-dbsearch-wf	https://tinyurl.com/ipepgen-dbsearch-input	https://tinyurl.com/ipepgen-dbsearch-history	https://tinyurl.com/discovery-gtn

3. Verify PSMs and novel neoantigen candidates	Verifies neoantigen peptide sequences identified in Module 2 using a secondary spectral matching algorithm, PepQuery2	https://tinyurl.com/ipepgen-pepquery-wf	https://tinyurl.com/ipepgen-pepquery-input	https://tinyurl.com/ipepgen-pepquery-history	https://tinyurl.com/verification-gtn
4. Annotate, classify, and visualize verified neoantigen sequences	Maps selected neoantigen peptide sequences to genes and transcripts, classifies the nature of non-reference sequences, and enables visualization	https://tinyurl.com/ipepgen-pep-annot-wf	https://tinyurl.com/ipepgen-pep-annot-input	https://tinyurl.com/ipepgen-pep-annot-history	https://tinyurl.com/peptide-annotation-gtn
5. Prioritize neoantigens for further study	Predicts binding of peptides to the HLA alleles and assesses potential immunogenicity, prioritizing candidates for further experimental evaluation	***HLA allele genotyping*** https://tinyurl.com/ipepgen-hla-genotyping-wf	***HLA allele genotyping*** https://tinyurl.com/ipepgen-hla-genotyping-input	***HLA allele genotyping*** https://tinyurl.com/ipepgen-hla-genotyping-history	https://tinyurl.com/hla-genotyping-gtn
		Peptide-HLA binding prediction https://tinyurl.com/ipepgen-iedb-pep-wf	***Peptide-HLA binding prediction*** https://tinyurl.com/ipepgen-iedb-pep-input	***Peptide-HLA binding prediction*** https://tinyurl.com/ipepgen-iedb-pep-history	https://tinyurl.com/binding-prediction-gtn

Online, on-demand, and interactive training resources lower barriers for new users. The iPepGen pipeline takes advantage of the powerful GTN [56], which offers online, on-demand, and interactive training resources for new users. The GTN is seamlessly connected with workflows and software available on public Galaxy servers, thus providing a means for new users to adopt sophisticated analysis pipelines for use in their studies. We developed an accessible "Learning Pathway" [58] within the GTN, which summarizes training resources for each module in the iPepGen pipeline, and leads users to the online documentation. The last column of **Table 1** shows the links to training materials that guide new users step-by-step through each workflow, complete with links to demonstration data and software. Once a user has completed these training workflows, they are equipped with the necessary knowledge to upload their data to their Galaxy account and begin their own meaningful immunopeptidogenomic analysis.

Description of analysis modules and representative results. We describe the main functionalities and operation of each module in the iPepGen pipeline, along with results from MPNST data that demonstrate its effectiveness for immunopeptidogenomic analysis. The

Methods section provides a more detailed description of software tools and key settings used in each module.

Module 1: Prediction of non-reference neoantigen peptide sequences

To demonstrate the functionality and output of the iPepGen pipeline, we analyzed RNA-Seq and immunopeptidomics data from an MPNST cell line. These demonstration analyses used the workflows and input data shown in **Table 1** and produced the analysis histories shown in this table as well. For Module 1, we utilize several complementary workflows seeking to cast a wide net for possible genomic or transcriptomic sequences that may code for novel proteoforms leading to neoantigen production and may be identified via matching to immunopeptidomic MS/MS data in downstream modules.

Gene Fusion Variant Detection. The first workflow targets gene fusions, a potential source of neoantigen peptides in many cancers [1], using the Arriba algorithm [59], integrated with the RNA STAR aligner [60] for spliced alignment and chimeric read detection. Outputs include a tabulated list of high-confidence fusions with supporting read metrics, visual summaries of each event in PDF format, and translated fusion junction sequences in FASTA format for downstream use (**Table 1**, Module 1, Gene Fusion neoantigens Item 21 in the history). Post-processing steps are applied to remove potential false positives based on read depth, mapping quality, and known benign fusion sequences. For the MPNST cell line RNA-Seq data used for demonstration, we identified 86 novel fusion sequences that were translated into potential neoantigen peptide sequences and outputted in the annotated FASTA-formatted table (**Figure 2**).

Comprehensive Prediction of Non-Reference Protein Sequences. Complementing the targeted prediction of gene fusion-derived neoantigens, we employed additional workflows that broadly identify non-reference transcript sequences from RNA-Seq data. In one of these workflows, paired-end reads are aligned to the human reference genome using HISAT2 [61], followed by variant calling with FreeBayes. CustomProDB [62] then annotates predicted coding DNA sequence (CDS) variants, generating a set of non-reference protein sequences containing single amino acid variants (SAVs) and short insertion-deletions (InDels).

In the second workflow, transcript assembly is performed using StringTie [63], with transcript classification via GffCompare [64] to distinguish novel sequences compared to the reference genome. Novel, non-reference sequences, including frameshifts, novel splice events, and/or sequences from non-coding genomic regions, are translated in all three reading frames using Galaxy tools “Translate BED Transcripts” and “bed to protein map.”

The peptide sequences predicted from fusions, SAVs/InDels, and other non-reference transcripts are all merged with UniProt canonical human protein sequences and common

contaminant sequences (called the common Repository of Adventitious Proteins, cRAP) using "FASTA Merge Files and Filter Unique Sequences." Non-reference entries are annotated with a "Generic" header, distinguishing them from UniProt canonical sequences, to support downstream analysis in Module 2. For the RNA-Seq from the MPNST STS-26T cell line, these combined workflows generated a protein database containing approximately 2.9 million unique protein sequences (**Table 1**, Module 1, Item 41 in history). **Figure 2** shows the FASTA-formatted databases generated by each of the three described workflows used for the prediction of neoantigen peptides in this module using this MPNST demonstration data. Notably, the cumulative number of entries across all individual databases exceeds that of the comprehensive database, as redundant or overlapping peptide sequences present in multiple workflow-specific outputs are collapsed into unique entries during the database merging process, thereby reducing redundancy in the final merged FASTA database.

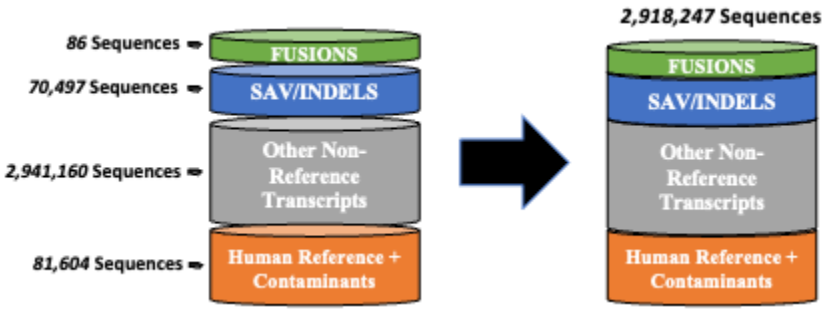

Figure 2. Relative size of databases from MPNST cell line RNA-Seq data generated by Arriba (fusions), CustomProDB (SAV/Indels) and StringTie (other non-reference sequences) workflows. The graphic illustrates the cumulative size of each non-reference database component and the final customized database after merging with the reference and common contaminant (cRAP) protein sequences and after removing redundant sequence entries.

Module 2: Discovery of candidate neoantigen peptide sequences and tumor-associated antigens

To identify candidate neoantigens presented by HLA class I molecules, we analyzed MS/MS immunopeptidomics data from the same MPNST cell line used in Module 1. This module utilizes the FragPipe software suite [65], which is deployed as a tool in Galaxy. We matched MS/MS spectra against the customized FASTA database of ~2.9 million sequences generated previously (**Figure 2**). The workflow integrates MSFragger for PSM generation, with MSBooster rescoring [66], Percolator [67] for post-search validation, and Philosopher [68] for downstream filtering and false discovery rate (FDR) control at 1% for both PSM and protein levels. FragPipe also offers the IonQuant tool [46] for label-free quantification (LFQ), quantifying identified peptide abundance based on their normalized LC-MS intensities. The "Nonspecific-HLA" search mode in FragPipe is used to identify HLA I-bound peptides ranging from 8 to 20 amino acids in length. To

accommodate the large search space when using no enzyme specificity, the workflow automatically partitions the protein database into 200 random sections for efficient parallel processing. For more efficient use of FragPipe in iPepGen, we developed a Galaxy tool called "Manifest Generator" for generating the tabular manifest experimental design file, which annotates input raw MS/MS scan files being analyzed into experimental groups defined by the user (e.g., control, treated). This file is required by FragPipe for operation and provides a comparison of LFQ values of identified peptides across sample groups, presented in the tabular output of all identified peptides from the analysis.

After the FragPipe analysis is complete, the Query Tabular Galaxy tool [69] extracts peptide sequences from the tabular outputs of FragPipe that did not match any canonical reference proteins, generating a new tabular file of candidate neoantigen peptides for further analysis. Users may further stratify these peptides by variant type (e.g., SAVs, InDels, gene fusions, etc.) by filtering on tag-based sequence annotations embedded during FASTA construction in Module 1. Using a single MS/MS file from the MPNST cell line STS-26T, and the comprehensive sequence database generated in Module 1, we identified 123 unique peptide sequences that mapped exclusively to non-reference proteins (**Table 1**, Module 2, Item 21 in history).

One key aspect of immunopeptidomics is the requirement to identify peptides using non-specific enzyme settings, as opposed to traditional bottom-up proteomics, which is based on analysis of peptides generated using proteolysis with trypsin. With non-specific enzyme settings, the possible sequence search space increases significantly, especially when using large databases including non-reference protein sequences predicted from NGS data. This challenges sequence database search algorithms to sensitively and efficiently generate PSMs for neoantigen discovery. FragPipe offers an ultrafast algorithm, which, when coupled with deployment in Galaxy on scalable compute resources, overcomes these limitations. As a demonstration, we compared FragPipe with the popular MaxQuant software, used in some available immunopeptidomics pipelines [50], evaluating their ability to identify HLA 1-bound peptides using non-specific enzyme settings (**Figure 3A**). Both tools were run on equivalent compute environments (16 cores, 64 GB memory). We limited the size of the customized protein sequence database to 250,000 entries because MaxQuant would not reliably run with larger databases, further demonstrating its limitations for neoantigen discovery. FragPipe identified a greater number of peptides at a global FDR of 1% while requiring significantly less runtime. This performance advantage can be attributed to MSFragger's ultrafast search capabilities, including database splitting, and enhanced scoring strategies and post-processing methods [70], making it particularly well-suited for high-throughput neoantigen discovery. We also benchmarked the performance of FragPipe based on the size of the input sequence database. Unsurprisingly, the analysis time and memory required scale with the size of the

database, with FragPipe generating results on even the very large 2.9M sequence database in under two hours on the publicly available European Galaxy server (see **Supplemental Table 1**).

In addition to MaxQuant, we also compared FragPipe's performance for no enzyme PSM generation from large sequence databases to the commercial software PEAKS, an efficient algorithm for PSM generation that has also been used in previously described immunopeptidomics pipelines [42]. FragPipe identified 30% more peptides than PEAKS for database searching against our large demonstration database containing over 2.9M sequences (See **Supplemental Figure 1**).

We also evaluated the effects of using the MSBooster algorithm [66] in FragPipe, which utilizes machine-learning-based prediction of fragment ion intensities and retention times, followed by a re-scoring of potential PSMs to increase confident peptide identifications. **Figure 3B** shows the use of MSBooster on our example immunopeptidomics dataset from MPNST cells, generating PSMs against the large protein sequence database. MSBooster significantly increases the number of confident PSMs generated, including identification of candidate neoantigen sequences. We have also tested the use of MSBooster on other datasets, where it showed an increase in total peptides identified of about 35% on average, and an increase of about 50% in identified non-reference sequences (**Supplemental Figure 2**).

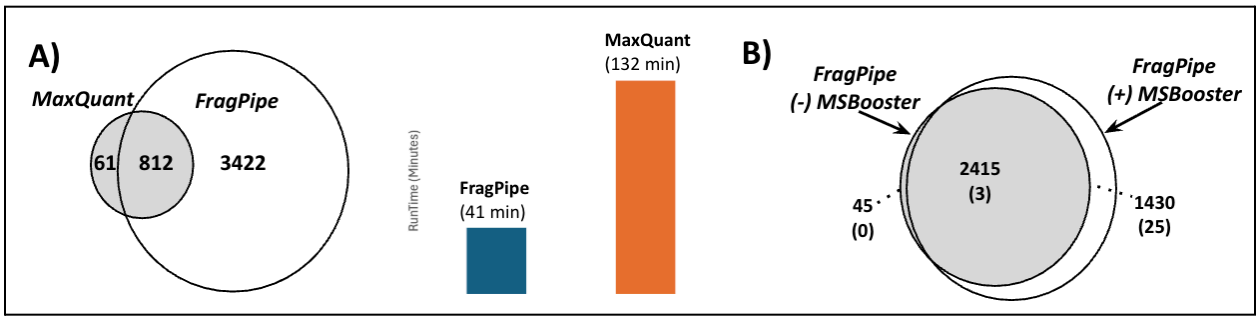

Figure 3. A. Comparison of FragPipe and MaxQuant performance for a "no enzyme" database search of a single peptidomics MS/MS file against an immunopeptidomics proteome database of 250K sequences from MPNST data in Module 1; both software were run using their stand-alone GUI using 16 cores and 64GB memory. **B.** Comparison of FragPipe run without and with MSBooster functionality on a single immunopeptidomics MS/MS datafile from MPNST cells and searched against a sequence database of 2.9M+ sequences, including the reference and annotated non-reference candidate neoantigen sequences; number of unique peptides identified are shown, with numbers in parentheses () indicating the number of candidate neoantigen peptides identified with sequences that do not map to the reference proteome.

Module 3: Verification of neoantigen peptide-spectrum matches

To further evaluate candidate neoantigens identified in Module 2, we implemented a secondary peptide-centric verification step using PepQuery2 [71]. This approach addresses the potential for false-positive PSMs when searching large, non-reference databases, as previously noted in

immunopeptidomics studies [72]. PepQuery2 allows for independent evaluation of individual PSMs by scoring candidate neoantigen sequences against MS/MS spectra in the presence of competing reference peptides, including common PTMs. This module also uses the BLAST-P tool [73] for verifying that neoantigen candidates are truly novel and do not match any known reference sequences. As such, the module provides a secondary verification of candidate neoantigens identified using FragPipe against the large sequence database to ensure users are provided the most confident and high-value peptides to consider for further study.

For the demonstration MPNST immunopeptidomics dataset, we evaluated the 123 candidate neoantigen peptides from Module 2 using PepQuery2. Nineteen neoantigen peptide sequences passed PepQuery2 hyperscore validation and BLAST-P filtering (**Table 1**, Module 3, Item 12 in history). BLAST-P alignment was performed at less than 100% sequence identity to canonical proteins to capture sequence variants and polymorphisms. These peptides represent the most confident neoantigen candidates ready for further characterization and prioritization for experimental evaluation. We include **Supplemental Figure 3,** which shows the multiple steps our pipeline utilizes in order to discover neoantigen candidates and verify those of highest confidence, generating a stringently filtered set of peptides for further assessment.

Module 4: Visualization and classification of verified neoantigen sequences

This module facilitates the classification and genomic annotation of verified neoantigen peptides. The PepPointer tool [74] is used to map neoantigen peptides to their genomic coordinates using BED and GTF annotations and classify their genomic origin as coding (CDS), untranslated region (UTR), intron retaining, or intergenic sequences. PepPointer outputs a tabular file with a description of their likely genomic origin, as well as genomic coordinates to use with the Integrated Genome Viewer (IGV) [75] and a URL for direct input into the UCSC Genome Browser [76], enabling researchers to inspect peptide-transcript-genome alignments visually. **Figure 4** shows an example output of a neoantigen candidate peptide from MPNST, visualized in IGV and mapped to the genome along with supporting assembled transcript sequences, generated in Module 1, that led to the predicted sequence that was verified in Modules 2 and 3.

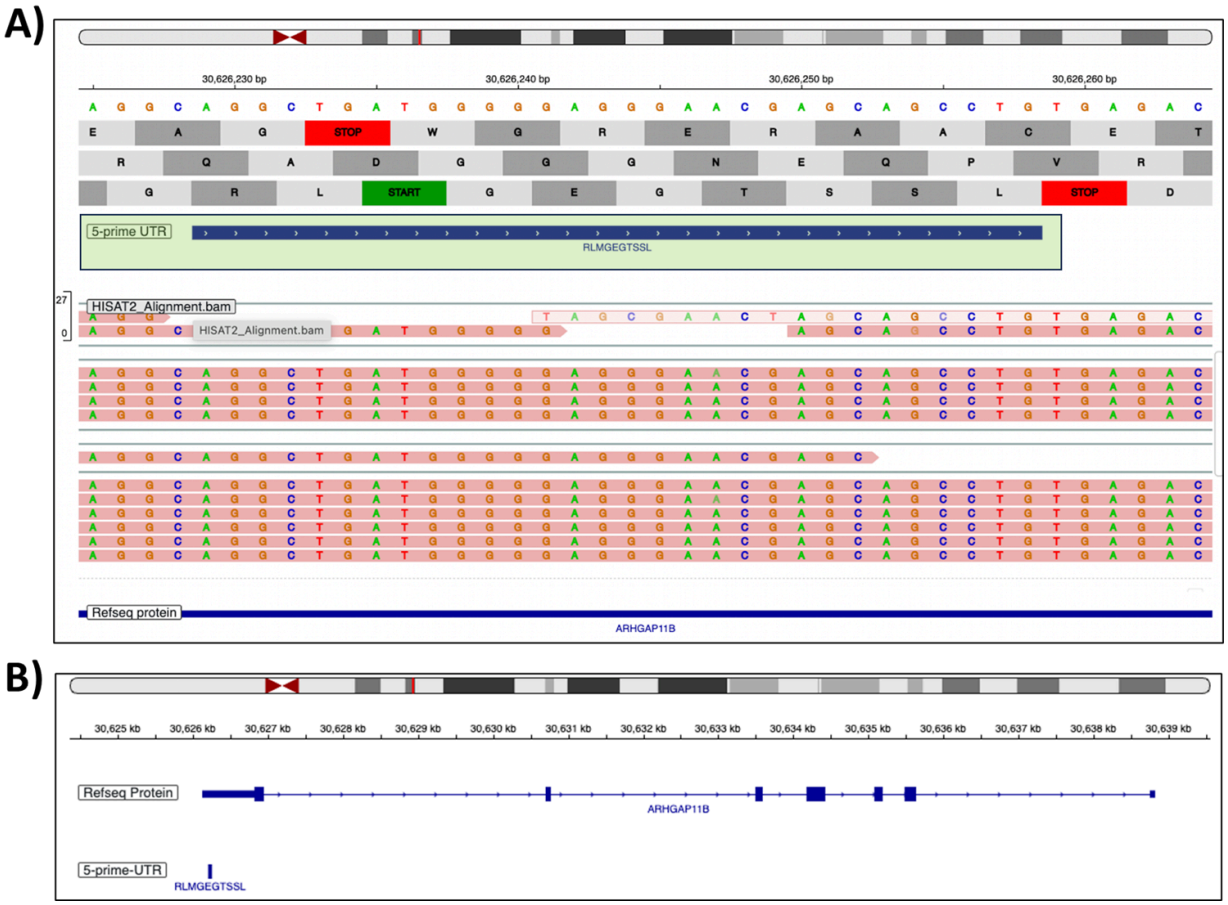

Figure 4. Example of a non-reference neoantigen peptide sequence (RLMGEGTSSL, shown in green shaded box) coded by a transcript sequence mapped to a portion of the 5' UTR of an annotated gene structure and visualized in the Integrated Genome Viewer (IGV). Assembled RNA-Seq reads coding this peptide are in pink; B) A larger view of the annotated protein coded by this genomic location (exon coding sequences shown in blocked blue) with the location of the novel neoantigen peptide mapped to the 5' UTR also shown as a reference.

Visualization in the UCSC browser using the URL generated by PepPointer offers a variety of tools for assessing the nature of neoantigen candidates and their potential for immunotherapy development. Visualization tracks can be easily added to assess gene expression in normal and cancerous tissues from the Genotype-Tissue Expression (GTEx) database [77], as well as gene expression profiles in cancerous tissues through resources such as Clinical Interpretation of Variants in Cancer (CIViC) [78] and the Cancer Genome Atlas (TCGA) cancer expression database [79]. **Supplemental Figure 4** shows an example of the same verified neoantigen peptide candidate shown in **Figure 4**, but visualized in the UCSC browser using some of these functionalities. Moreover, the tabular output provides users an easy means to input verified neoantigen peptide sequences into other resources, such as the PeptideAtlas resource [80], to

determine if a peptide has been identified in other published MS-based peptidomics studies, or other repositories of neoantigens identified in prior, published studies [81,82].

In some cases, candidate neoantigen peptides arise from transcript coding sequences derived from complex processing of DNA or RNA sequences (e.g., unique combinations of intron and exon sequences), which are not easily annotated via PepPointer and may require manual exploration by the user for their advanced characterization. **Supplemental Figure 5** outlines one such example found in the nineteen neoantigen candidates outputted in Module 4 for the MPNST demonstration data (peptide HSEVQTLKY). Although this mapped to an intronic region, it required more manual investigation. **Table 2** summarizes the panel of nineteen verified neoantigen peptides from the demonstration data. These peptides were supported by high FragPipe PSM score probabilities (≥0.95) and deemed confident by PepQuery2 verification with highly significant p-values (≤1.0E-03). Notably, peptides in **Table 2** were derived from both canonical (CDS, UTR) and non-canonical genomic coding regions (introns, intergenic), underscoring the potential of iPepGen to uncover promising neoantigen peptides from diverse genomic and/or transcriptomic aberrations.

Table 2. Verified and classified neoantigen peptides from MPNST demonstration data.

Peptide	Protein	Genomic location	Strand	Annotation	FragPipe Probability	PepQuery p-value	HLA binding (Weak)	HLA binding (Strong)
APAPAAARLG	myosin binding protein C, cardiac	chr20:45834217-45834247	+	CDS	0.9749	1.04E-03	HLA-B*08:01 (partial) HLA-C*05:01 (partial) HLA-C*07:01 (partial)	
EASGALLRA	Chain A, Ezrin-radixin-moesin-binding phosphoprotein 50	chr3:111932306-111932333	+	CDS	0.9636	3.15E-04		
FLARKPSAV	40S ribosomal protein S14	chr5:150447594-150447567	-	CDS	0.9663	1.27E-04	HLA-C*05:01 HLA-C*07:01	HLA-A*02:01 HLA-B*08:01
HHGQPGVGI	pre-mRNA cleavage complex 2 protein Pcf11 isoform 1	chrX:63724661-63724634	-	CDS	0.9814	4.27E-03		
HSEVQTLKY	spindle and	chr13:211763	-	intron	0.9852	1.13E-04	HLA-C*05:01	HLA-A*01:01

	kinetochore -associated protein 3 isoform 1	36-21176309					HLA-C*07:01	
IRTATGPAKLRNSL	TOG array regulator of axonemal microtubules protein 1 isoform 2	chr3:196058601-196058559	-	CDS	0.9704	1.00E-04	HLA-C*07:01 (partial)	
ISDPGVQGY	type XIII collagen	chr9:108933791-108933764	-	exon	0.9926	1.52E-03	HLA-B*44:02 HLA-B*44:27	HLA-A*01:01 HLA-C*05:01 HLA-C*07:01
ITEVQQELY	ring finger protein 135	chr8:60572150-60572177	+	intron	0.9958	4.73E-04	HLA-C*05:01 HLA-C*07:01	HLA-A*01:01
LTDSAREMGY	zinc finger protein 717 isoform X1	chrUn_GL000218v1:96830-96800	-	Complex - requires manual interpretation	0.9609	2.03E-04		HLA-A*01:01
MADRVSAGF	inducible nitric oxide synthase	chr22:40177989-40178016	+	five_prime_utr	0.9624	1.27E-04	HLA-A*01:01	HLA-C*05:01
NTDRPGGYSY	immunoglobulin heavy chain junction region	chr12:123230271-123230241	-	CDS	0.9751	2.06E-04	HLA-C*05:01 HLA-C*07:01 HLA-B*44:02 HLA-B*44:03 HLA-B*44:27	HLA-A*01:01
NTESFGTGIK	unnamed protein product	chr5:60944940-60944910	-	CDS	0.9429	1.06E-04	HLA-A*01:01 (partial) HLA-C*05:01 (partial)	
RLMGEGTSSL	Rho GTPase-activating protein 11A	chr15:30626228-30626258	+	five_prime_utr	0.9893	1.06E-04		HLA-A*02:01
RPTENETAL	mitogen-activated protein kinase kinase kinase kinase 5 isoform X1	chr10:6580656-6580683	+	intergene	0.9916	3.15E-04	HLA-C*05:01 HLA-B*08:01	
RTDQFYVVY	EF-hand domain (C-terminal) containing 2, isoform CRA_b	chr12:56672720-56672693	-	intron	0.9749	5.07E-04		HLA-A*01:01 HLA-C*05:01 HLA-C*07:01

VSDSKSSSLY	immunoglobulin heavy chain junction region	chr6:53794404-53794374	-	intergene	0.9636	1.58E-03		HLA-A*01:01
YLDPAQQNLY	zinc finger protein 519, isoform CRA_c, partial	chr1:247201961-247201931	-	intergene	0.9663	3.17E-04	HLA-C*07:01 HLA-B*44:02 HLA-B*44:27	HLA-A*01:01
YVNEKRYPL	protein kinase NYD-SP5	chr20:25195875-25195902	+	intron	0.9814	1.27E-04	HLA-C*05:01 HLA-A*02:01	HLA-B*08:01 HLA-C*07:01
FLDKNDEYMKY	Fucosyltransferase 11 (alpha (1,3) fucosyltransferase)	chr10:73773347-73773380	+	CDS	0.9852	1.00E-04	HLA-C*05:01	HLA-A*01:01

Module 5: Prioritization of neoantigens for further study

With verified neoantigen peptides in hand from the modules above, iPepGen offers a module to assist users in prioritizing those sequences that may be of highest interest for further evaluation in the lab. This prioritization is guided using software for predicting binding and potential recognition as immunogenic epitopes that may be of highest value for immunotherapies. Two workflows make up this module to enable this prioritization.

HLA Genotyping. As a first step to predicting peptide binding to the MHC (or HLA for human samples), allele genotyping is performed on RNA-seq data. Paired-end reads in FASTQ format are analyzed using two complementary tools: OptiType [83], optimized for class I alleles, and Seq2HLA [84], which also covers class II loci, if desired. Outputs from the two tools are merged, and redundant entries are removed using Galaxy text manipulation tools. In the case of MPNST RNA-Seq data used for demonstration purposes, the final genotype set included eight HLA class I alleles (**Table 1**, Module 5, Item 13 in first history), which served as input for further neoantigen peptide-binding affinity prediction.

Peptide–HLA Binding Prediction. The genotyping results are used as one input to the IEDB MHC Binding Prediction tool [85], using the NetMHCpan_EL algorithm, trained on empirical immunopeptidomics data. The neoantigen peptides from Module 3 are used as the other input. If the input peptides are 9 or more amino acids long, the software generates overlapping shorter sequences of at least 8 amino acids and evaluates each for HLA binding from human samples, thereby expanding the list of potential peptide binders beyond the original input set. Peptide sequences are assigned a percentile rank for their binding to each HLA allele and classified as strong (percentile rank ≤ 0.5%) or weak binders (0.5 – 2%). Binding results for the

neoantigen candidates identified from the MPNST dataset are shown in **Table 2**. Thirteen peptides were predicted as strong binders (**Table 1**, Module 5, Item 12 in the second history), and ten were classified as weak binders (**Table 1**, Module 5, Item 11 in the second history) to specific HLA alleles. The specific combination of predicted binding affinity to specific HLA alleles can be used as an indicator of immune recognition [86] and provides a means to prioritize peptides for further experimental evaluation.

Enhanced prioritization and immunogenicity prediction. The outputted HLA allele genotypes and verified neoantigen candidate peptide sequences from iPepGen serve as input to the pVACbind software suite, which we deployed as a Galaxy tool. pVACbind is part of the personalized Variant Antigens in Cancer tool suite (pVACtools) [24]. The pVACbind software bundles numerous complementary tools for predicting binding of input peptide sequences to HLA protein complexes (both class I and II) coded by specific allele genotypes, as well as two deep learning algorithms, DeepImmuno [29] and BigMHC [30], for predicting immunogenicity of HLA alleles and bound peptides. The Galaxy pVACbind tool outputs a tabular file with aggregated results across all the algorithms selected by the user, as well as a filtered output showing only those peptide and allele combination scores indicating high potential for immunogenicity. The pVACbind software is well documented [87] and provides users with ample information about the bundled tools and how to interpret results for assisting in prioritizing peptides for further experimental testing as immunotherapy agents. We have documented the use of pVACbind in Galaxy in the GTN, and also in **Supplemental Figure 6 (a, b, c)**.

Extended applications of the modular iPepGen pipeline

An end-to-end "One-Click" iPepGen workflow. We have intentionally used a modular design in developing the iPepGen pipeline, as described above in detail. This design provides the best means to develop module-specific online training material accessible through the GTN, aimed at training new users seeking to adopt these tools and apply them to their own data. However, for more advanced users who have trained through the GTN, a module-by-module analysis may be cumbersome, especially for studies generating multiple MS-based immunopeptidomics datasets requiring reproducible analysis [88]. To this end, we developed a comprehensive workflow integrating all the modules of iPepGen. In this embodiment, the user simply uploads their starting raw data (e.g., paired RNA-Seq, LC-MS/MS scan files) along with the required reference proteome sequence database, genome sequence annotation files, and manifest experimental design file. The entire pipeline is instantiated with a single click of the "Run" button for the workflow. Although all intermediate files are recorded and saved in the resulting Galaxy analysis

history, we have simplified the outputs of this integrated workflow so only those of the highest value for interpreting results are exposed to the user in the generated history.

We developed the one-click workflow using our single MPNST dataset. To further demonstrate its effectiveness, we utilized publicly available data from a recent immunopeptidogenomic study by the Purcell group [89]. RNA-Seq data from an acute myeloid leukemia cell line were selected along with three raw MS/MS files of technical replicates from an experiment testing an HLA class I immunopeptidomic enrichment method. Links to these analysis histories, as well as the one-click workflow itself, are shown in **Table 3** below. Analysis of the published data generated six verified neoantigen candidate peptides predicted from String-Tie identified non-reference sequences (Item 153 in the shared analysis history), but none from CDS sequences (SAVs or InDels).

Flexibility of iPepGen: building customized workflows for neoantigen identification and verification, and PTM analysis. Galaxy provides the unique flexibility to integrate selected modules of iPepGen into customized workflows tailored to specific analysis applications. One use-case requiring iPepGen customization would be scenarios where a customized protein FASTA database (containing potential non-reference neoantigen sequences) has been generated externally, and PSM discovery and verification from MS/MS data is required. For such a situation, we built a customized workflow that integrates Modules 2 and 3 for neoantigen discovery and verification, respectively.

To demonstrate the effectiveness of this customized iPepGen workflow, we selected data from a published study by the Bassani-Sternberg group focused on neoantigen discovery in a melanoma cell line [90]. We selected four biological replicate immunopeptidomics raw MS/MS datasets generated from untreated control melanoma cells and four raw MS/MS files from biological replicates of cells treated with 5-aza-2′-deoxycytidine (DAC), which promotes processing and presentation of peptide antigens on the HLA class I complex. These raw MS/MS datasets were generated using optimized immunopeptide enrichment and MS instrumental analysis methods, such that they represented extremely data-rich MS/MS files for demonstrating the effectiveness of this customized workflow. A customized protein sequence database of about 900,000 sequences made available in the published data, was used. It contained predicted, novel proteoform sequences from long-noncoding RNA, transposable elements, and other non-reference sequences, including SAVs and other CDS mutations, annotated from RNA-Seq data on these cell lines. Analysis by our customized pipeline identified 22,386 distinct peptides using FragPipe across these eight MS/MS files in Module 2, complete with LFQ abundance levels (item 49 in the linked analysis history in **Table 3**). This deep dataset also generated several hundred potential neoantigen candidates, which were advanced to the

verification Module using PepQuery2 and BLASTP. 93 distinct neoantigen candidate peptides were verified (item 85 in the linked analysis history in **Table 3).** We were able to quantify neoantigen candidates and their response to DAC treatment, as well as known TAAs derived from reference human protein sequences. **Supplemental Figure 7** shows example results for a verified neoantigen peptide and a known melanoma TAA, which showed an expected increased abundance with DAC treatment.

We also used this customized workflow to demonstrate the ability of the iPepGen pipeline to identify HLA-bound peptides carrying PTMs that may regulate peptide processing for presentation to the immune system. For this demonstration, we focused on cysteine glutathionylation, known to regulate HLA presentation [91]. We used six MS/MS datafiles from the same published study analyzing melanoma cells [90], and added cysteine glutathionylation as a variable modification for the FragPipe analysis. This resulted in the identification of 105 peptides containing glutathionylated cysteine (item 41 in the linked history in **Table 3**), complete with LFQ abundance measures using IonQuant in FragPipe. These candidates were then passed on to PepQuery2 for verification. Here, PepQuery2 was operated to evaluate "known" sequences in the human reference proteome, including those with a variable modification to cysteine. PepQuery verified 28 distinct cysteine glutathionylated (item 64, the linked analysis history in **Table 3**).

Table 3. Links to workflows and demonstration analysis histories for the One-Click Workflow and Customized Workflows

Application	**Workflow**	**History**
One-Click Workflow (Complete iPepGen pipeline)	i) **Single sample** https://tinyurl.com/ipepgen-oneclick-wf ii) **Dataset collection** https://tinyurl.com/coll-ipepgen-oneclick-wf	i) **Single sample** https://tinyurl.com/ipepgen-oneclick-history ii) **Dataset collection** https://tinyurl.com/coll-ipepgen-oneclick-history
Customized neoantigen discovery and verification workflow (Modules 2 and 3)	https://tinyurl.com/ipepgen-disc-verify	https://tinyurl.com/ipepgen-disc-verify-history
Customized PTM-peptide discovery and verification workflow (Modules 2 and 3, cysteine glutathionylation example)	https://tinyurl.com/ipepgen-disc-verify-ptm	https://tinyurl.com/ipepgen-disc-ver-ptm-history

Discussion

iPepGen's design addresses key challenges unique to immunopeptidomics, including the use of personalized protein databases derived from NGS data, peptide identification using no enzyme specificity, verification of non-reference peptide sequences, and computational prioritization of candidate neoantigens. Several other informatics platforms aimed at complete immunopepetidogenomic analysis have also been described [42,47,48], notably the NeoDisc [51] pipeline, which offers a sophisticated set of tools including a deep learning algorithm to prioritize candidate neoantigens with the highest potential for value in immunotherapy. iPepGen offers similar capabilities to these available platforms, but also several distinguishing features:

- iPepGen implementation in the Galaxy ecosystem, featuring accessibility on publicly available, scalable cloud-computing infrastructure, enables usage by those who lack software developer expertise, and/or access to HPC computing environments required for compute-intensive applications inherent to immunopeptidogenomics. This contrasts with platforms such as NeoDisc, which, despite offering very powerful capabilities including the use of FragPipe for PSM discovery, require advanced technical know-how and access to local HPC infrastructure for implementation and maintenance of its operation, limiting its use by those lacking this expertise and computer hardware.
- The availability of online training material through the GTN empowers adoption by users with limited computational expertise, providing a step-by-step guide to learn each module of the iPepGen pipeline in a hands-on manner, using supplied demonstration data. Once trained, these users can advance to using the One-Click workflow for analysis of their own datasets.
- Trained users can take advantage of the transparent and flexible features of the Galaxy analysis environment to customize their own analyses, using the described iPepGen modules as a foundation. Users can take advantage of the rich library of training material offered by the GTN [56,57] to integrate new software tools (e.g., additional tools to predict novel proteoforms from genomic data) or build customized workflows tailored to their analysis needs. Adaptability to new software integration is a hallmark of the Galaxy ecosystem, which also offers a thriving community of developers and users, ensuring sustained maintenance and operation [53]. Other platforms, such as NeoDisc, do not offer such flexibility and require advanced software development expertise to customize the pipeline and maintain its operation.

In **Module 1**, we have developed several workflows to assemble and annotate a diverse profile of non-reference genomic and transcriptomic sequences that may give rise to novel proteoforms, which, in turn, are potentially processed into neoantigen peptides. We have developed these workflows to build protein databases personalized to the user's sample(s) with a broad profile of potential neoantigen sequences, which are confirmed via integration with

MS/MS data in Module 2. This module is flexible to customization with other approaches to identifying specific types of non-reference genomic and/or transcriptomic sequences from NGS data, including single-cell, long-read sequencing, or other “third-generation sequencing” methods [92]. Galaxy has a well-established history as an environment amenable to the integration of emerging software for genomic and transcriptomic sequencing analysis [93]. iPepGen offers compatibility with a wide array of analysis tools in this area, providing a means to build protein sequence databases containing potential neoantigen sequences for interrogation with immunopeptidomic MS/MS data.

The use of FragPipe in **Module 2**, and specifically the MSFragger algorithm, provides the backbone for matching immunopeptidomic MS/MS data to the customized sequence databases from Module 1. MSFragger’s ability to efficiently handle "no enzyme" search settings is critical in this context, as MHC-bound peptides are often produced by a variety of proteases, unlike typical proteomic samples that are digested with trypsin. The expanded search space inherent to immunopeptidomic data substantially increases memory and processing requirements, especially when searching against a custom database containing over 2 million protein sequences. To accommodate this, we enabled MSFragger’s “split database” functionality, dividing the database into 200 sections. Deployment of iPepGen in a scalable, cloud-based Galaxy instance is also key. The European Galaxy instance housing our workflows allocates up to 250 GB of active memory, which proved sufficient for executing MSFragger under these conditions.

We also evaluated the MSBooster tool [66] in FragPipe, which utilizes machine-learning-based scoring to predict fragment ion intensities and retention times. After this prediction, potential PSMs are re-evaluated, which results in the rescue of many peptide identifications and enhances sensitivity. In our analysis of representative datasets (**Figure 3B** and **Supplemental Figure 2**), incorporating MSBooster significantly increased identification of peptides, both reference and non-reference neoantigen candidates, without extending runtime or memory consumption. iPepGen also takes advantage of FragPipe’s IonQuant module [46] for LFQ by aligning peptides across LC-MS/MS datasets and calculating abundances based on area-under-the-curve measurements and match-between-run functionality. This feature is enabled in our shared workflows and allows researchers to incorporate abundance measurements of identified neoantigen peptides and compare their response across different experimental conditions.

In **Module 3**, PepQuery2 offers a peptide-centric means to verify candidate neoantigen sequences through a secondary analysis of peptides discovered in Module 2. Unlike conventional sequence database search tools, PepQuery2 focuses exclusively on user-defined candidate neoantigen peptide sequences and evaluates their match quality to potential MS/MS

spectra in the dataset against human reference peptides, while also accounting for common PTMs to these peptides. Our use of PepQuery2 is another distinguishing feature of iPepGen, as other pipelines rely on traditional sequence database searching to identify neoantigen candidates and lack such a stringent verification step as that offered by PepQuery2. We did observe that using non-specific enzyme digestion increases memory demands for PepQuery2, such that limiting the amino acid length of reference peptides for consideration to a narrow window around the input neoantigen candidates was necessary for successful operation (see Methods for more details).

Module 4 provides an initial means to classify non-reference peptides based on the aberrant genomic or transcriptomic sequences coding for the identified sequence. The simple tabular output also provides links to be imported into the popular IGV or UCSC genome browser to visualize the genomic coordinates coding for the peptide, along with any supporting transcript sequences responsible for the translation product. As we note in the Results section describing this module, and **Supplemental Figure 5**, the complex nature of some aberrant genomic and/or transcriptomic sequences coding neoantigen peptides may require deeper manual investigation to understand the source of some peptide sequences. The ability to visualize the sequences coding for candidate neoantigen peptides in IGV or UCSC offers users guidance in characterizing these complex scenarios.

The HLA genotyping tools in **Module 5**, OptiType and Seq2HLA, provide complementary strengths for sequence analysis of HLA alleles from NGS data. OptiType is optimized for Class I alleles, while Seq2HLA genotypes both Class I and Class II alleles. Use of these two tools offers more comprehensive genotyping of HLA alleles. While Module 5 provides basic prioritization functionality using prediction of binding affinity to the genotyped alleles as a factor in immunogenicity, more advanced machine-learning-based tools are increasingly available to predict immunogenicity from multi-omics datasets [24,94–96]. With this in mind, we have implemented the pVACbind [87] software in Galaxy, part of the pVACtools software suite [24], compatible with the outputs generated by our pipeline. pVACbind provides complementary tools for predicting HLA binding, as well as two deep learning algorithms for predicting immunogenicity [29,30].

In addition to the availability of workflows and accompanying training material making up each module of iPepGen, we have also developed several customized versions of the pipeline. These demonstrate the flexibility offered by Galaxy implementation. One of these is the “One-click” workflow, which offers a more streamlined option for advanced users to upload appropriate raw data and generate results in a single click. We have demonstrated this workflow’s effectiveness on data available from a published immunopeptidogenomics study [89]. It should offer an easy-to-use workflow for users who have gained training via our GTN materials and are ready to

analyze their own data. We also developed a customized workflow that integrates Modules 2 and 3, for use-cases where a FASTA-formatted protein sequence database containing potential non-reference neoantigen sequences is already available, along with immunopeptidomics MS/MS data. We demonstrate the effectiveness of this workflow on deep immunopeptidomics data from a published study [90], showing the ability of iPepGen to efficiently handle larger volumes of data. Finally, we use this customized workflow and this same dataset to demonstrate the ability to identify and verify PTMs on HLA-bound peptides, using cysteine glutathionylation as an example. Collectively, these extensions of iPepGen provide a demonstration of the unique flexibility of the pipeline to adjust to the experimental needs of users.

Although iPepGen offers a rich set of functionalities required for immunopeptidogenomic data analysis, some additional features will be the focus of future work. We have focused our demonstration of its effectiveness on HLA class I immunopeptidomics data. However, the pipeline can be easily adapted to class II studies. Module 2 already generates PSMs for a broad range of peptide lengths, including the most common lengths (12-20 amino acids) for class II-bound peptides [97]. This length range can be modified in FragPipe as necessary if other peptide lengths are anticipated. In Module 3, the length range for PepQuery2 can be easily adjusted to verify peptides within this length range. Seq2HLA offers the ability to genotype HLA class II alleles, and IEDB tools in Galaxy can predict the binding of peptides to these alleles. Moreover, pVACbind provides several additional tools for class II peptide binding prediction, and includes the DeepImmuno tool [29] that predicts immunogenicity for class II peptide-allele pairs.

The current workflow focuses on the analysis of data-dependent acquisition (DDA) peptide MS/MS data. With the advent of new, faster scanning instruments, data-independent acquisition (DIA) data is increasingly used for immunopeptidomics [98,99]. DIA offers potential for deeper detection of peptides in complex mixtures and more accurate and precise quantification [100]. The FragPipe suite offers tools for DIA analysis [101,102], which are readily available for analysis of these data types in our pipeline. However, utilizing a large, customized sequence database and verifying neoantigen candidates discovered from DIA data offers challenges that will need to be addressed. Lastly, de novo sequencing from MS/MS data could offer identification of neoantigen peptide candidates that are not predicted from genomic or transcriptomic data [103], and could be explored for added value.

Conclusion

In summary, the iPepGen pipeline offers several distinguishing qualities from other available informatics solutions for immunopeptidogenomic informatics:

- Immediate access to analysis modules on a scalable cloud-based infrastructure, with the option to run individual modules or together as a complete pipeline for the discovery, verification, and prioritization of neoantigen peptides for further experimental evaluation.
- Step-by-step, interactive training material for each module is encapsulated in a customized learning pathway [58], seamlessly linked to accessible workflows, empowering new users with limited bioinformatic expertise or access to advanced computational resources to learn and use the pipeline for their studies.
- Workflows composed of tools selected to meet key challenges and common bottlenecks of neoantigen discovery and verification, offering efficient and sensitive identification of peptides processed by non-specific proteases and rigorous verification of candidate neoantigen peptides using two algorithms (FragPipe and PepQuery2).
- Extensible and customizable implementation, open to new software tools as they emerge, and development of customized workflows integrating selected modules of the pipeline to meet user analysis requirements.
- Promotion of reproducibility and transparency via sharing of customized workflows and completed analysis histories for use by others and/or as an accessible record to accompany method descriptions in peer-reviewed publications.

Taken together, these features of iPepGen make it a unique and timely resource for the growing community of researchers conducting immunopeptidogenomics analyses. It offers an important contribution to the immuno-oncology research community seeking to develop improved therapies for treating and preventing a wide range of cancers.

Methods

Aim, Design, and Setting of the Study

This study aimed to develop and validate iPepGen—a modular, end-to-end, Galaxy-based immunopeptidogenomic pipeline designed to enable accessible and reproducible identification, verification, and prioritization of neoantigen peptides from cancer multi-omics data. The design integrates genomic, transcriptomic, and mass spectrometry (MS)-based immunopeptidomics data. Development of each module and associated workflows, along with demonstration data analysis, was conducted on the European Galaxy server [104].

Description of Materials and Data

For data demonstrating the effectiveness of iPepGen analysis modules, we used the STS-26T malignant peripheral nerve sheath tumor (MPNST) cell line. Total RNA was extracted and sequenced on an Illumina platform, yielding 100 base-pair paired-end reads (~80 million

reads/sample). Cells were treated with gentamicin (50 µg/mL) to promote ribosomal read-through and enhance expression of non-reference proteoforms [105]. MHC class I complexes were immunoprecipitated using the W6/32 monoclonal antibody, and bound peptides were analyzed on an Orbitrap Eclipse mass spectrometer using data-dependent acquisition (DDA) mode [106,107]. Using RNA-Seq and immunopeptidomics data from an MPNST cell line as a representative of user data inputs and analysis requirements, we describe the functionality and show representative results demonstrating the effectiveness of the iPepGen pipeline. **Table 1** provides links to the raw input data generated from the MPNST cells, as well as completed analysis histories.

iPepGen Pipeline Modules

The links in Table 1 provide access to the Galaxy software tools composing workflows of each iPepGen module, along with validated and optimized settings for their operation. Here, we describe briefly the core tools used in each workflow and the settings for specific tools necessary for effective operation and performance.

Module 1: Prediction of Non-reference Neoantigen Peptides. Paired-end RNA-Seq reads are aligned to the GRCh38 reference genome using HISAT2 [61]. In one workflow, gene fusion events are identified using Arriba [59], which utilizes spliced alignments from STAR [60] to detect chimeric reads. For broader non-canonical transcript detection, including single-nucleotide variants (SAVs), indels, and aberrant transcripts, a workflow using FreeBayes [108] was employed for variant calling coupled with CustomProDB [62], generating a protein database of SAV and short InDel sequences. A parallel workflow uses StringTie [63] and GffCompare [64] for transcript assembly and annotation of non-reference sequences, followed by the use of the Galaxy tool "Translate BED transcripts" and "bed to protein map" to generate a customized FASTA database. For both the CustomProDB and StringTie-based workflows, permissive thresholds for Reads Per Kilobase per Million mapped reads (RPKM) were used (Value of 1 for CustomProDB and 2 for StringTie) to retain low-abundance transcripts that may encode tumor-specific proteoforms. Customized protein databases with predicted non-reference sequences are merged along with the human reference sequences from UniProt [109] and common contaminant proteins using the Galaxy tool “fasta_merge_files_and_filter_unique_sequences”, generating a comprehensive, annotated FASTA-formatted database of over 2.9M sequences for downstream analysis.

This module requires the installation and configuration of HISAT2, STAR, FreeBayes, StringTie, GffCompare, Arriba, and CustomProDB within Galaxy. Of these, FreeBayes is the most computationally intensive, requiring approximately 10 CPUs and at least 17 GB of RAM per sample. A single RNA-Seq dataset may take up to 30 hours to process. Other tools in the module

typically operate efficiently with 4–8 CPUs and 8–16 GB RAM, completing in less than 4–6 hours.

Module 2: Discovery of Candidate Neoantigen Peptides. In module 2, raw LC-MS/MS files (for demonstration data, .raw formatted files from the ThermoFisher Orbitrap instrument) can be converted to mzML format using msconvert [110] or directly analyzed in native format by the FragPipe software suite. For this module, FragPipe (v23.0) uses these software components: MSFragger [70] for sequence database searching under no-enzyme settings (allowing identification of peptides 8–20 amino acids long), MSBooster [66] for deep-learning-based prediction of fragmentation and retention time patterns, Percolator [67] for machine-learning-based PSM validation, and Philosopher [68] for false discovery rate (FDR) estimation using a target-decoy approach. For demonstration data from MPNST cells, MS/MS spectra generated on the Orbitrap LC-MS/MS system were matched to the merged protein FASTA database generated in Module 1, generating peptide spectrum matches (PSMs). For the demonstration data, only oxidation on methionine was included as a variable PTM. Non-reference peptides were first automatically selected based on lack of direct sequence match to a known reference protein provided in the tabular FragPipe peptide report, and then extracted with annotation using Galaxy's Query Tabular tool [69], based on header annotations in the FASTA file indicating their origin from the workflows in Module 1 (e.g., fusion, SAV/InDel, or novel transcript).

This module requires the Galaxy-deployed FragPipe suite, msconvert, and Query Tabular. PSM generation using non-specific enzyme settings against a large customized sequence database of several million entries is memory intensive. To address this challenge, it is important to configure FragPipe to use a database split of at least 200 to lessen the memory burden. Users could also disable the "Mass calibration and optimization" setting as well to save on memory load. Additionally, the Galaxy FragPipe tool was configured with an allocation of 16 CPUs and 250 GB RAM. Using these settings, a typical DDA file (~1 GB) was processed within 6 to 8 hours on the European Galaxy server.

Module 3: Verification of Neoantigen Peptide-Spectrum Matches. This module implements a second level of PSM validation using PepQuery2 [71]. Neoantigen candidate peptides identified in Module 2 input as a tabular list of sequences are re-evaluated against the raw MS/MS spectral data, converted to MGF format via msconvert, and the UniProt reference proteome. PepQuery2 conducts peptide-centric analysis by considering reference proteome sequences of close length and mass to the candidate neoantigen sequence, along with common PTMs (e.g., phosphorylation, deamidation, acetylation, etc.), as potentially competing matches to MS/MS spectra that led to the identification of the non-reference sequence. PepQuery also generates thousands of randomized sequences of similar amino acid length and mass and evaluates

potential matches to the MS/MS spectra under consideration. Through these evaluations, a hyperscore is assigned for sequences matching the MS/MS spectrum, and statistical modeling to assign p-values to the best sequence match to the spectrum is conducted. Verified neoantigen peptides are those that remain the top match to the MS/MS spectrum (as opposed to a reference sequence or a randomized sequence) and are assigned a p-value indicating strong statistical significance. PepQuery2 assigns a Yes or No Confidence to each input peptide based on these assigned values. In our workflow, neoantigen peptides deemed confident are further evaluated using BLAST-P against the NCBI reference database to confirm that these are truly novel sequences, with no exact matching sequences found in the reference proteome. We note that the non-redundant NCBI reference database is continuously updated, so the output novel sequences from this module may change over time with these updates.

To ensure the performance of PepQuery2, when considering peptides from non-specific enzyme processing, using narrow amino acid length windows for reference sequences to consider is critical. For example, for neoantigen candidate peptide sequences nine amino acids in length, a narrow window (e.g., 9-11 amino acids) is suggested to minimize the number of reference peptides that need to be generated initially by the software, controlling the active memory required. These settings provide the algorithm with thousands of reference peptides to consider as competing for matching to MS/MS. Only peptides falling into the amino acid length range selected by the user can be confidently verified, so any input peptides of other lengths should be ignored. Using these optimized conditions, PepQuery2 completed the evaluation of 123 candidate sequences in under 20 minutes, using ≤8 GB RAM and 2 CPUs. BLAST-P queries using the Galaxy tool were also completed in under 30 minutes using similar compute allocations, making this module both computationally tractable and scalable.

Module 4: Visualization and Classification of Neoantigen Origins. This module utilizes the PepPointer tool [74] to map non-reference neoantigen peptides to their genomic coordinates using BED and GTF annotations assigned to the sequences. It also classifies their origin as being from coding DNA sequence (CDS), untranslated regions (UTR), intron-retained, or other intergenic sequences. The tabular output provides links with necessary information to be input into the IGV [75] or the UCSC Genome Browser [76], enabling researchers to inspect peptide-transcript-genome alignments visually. Galaxy's Query Tabular tool was used to format, annotate, and extract relevant features from intermediate outputs and generate tabular results with formats compatible with PepPointer. The tools in this module are not compute-intensive, typically completing in <15 minutes using 1–2 CPUs and ≤4 GB RAM, even when analyzing multiple peptide entries.

Module 5: Prioritization of Neoantigens via HLA Binding Prediction. HLA genotyping is performed using two complementary tools: OptiType [83], optimized for class I alleles, and

Seq2HLA [84], which also covers class II loci. Paired RNA-Seq fastq files serve as input, and genotype outputs are merged using AWK in Galaxy and formatted via Query Tabular into tabular formats compatible with the IEDB MHC Binding Prediction tool [85]. The NetMHCpan_EL algorithm, trained on empirical data for eluted peptides from immunopeptidomics studies, predicts binding affinity of verified neoantigen peptides (which are provided as input) to specific HLA alleles. Peptides are classified as strong (binding affinity percentile rank ≤ 0.5%) or weak binders (percentile rank 0.5–2%) to specific HLA sequences. Peptides of 9 or more amino acids are processed into shorter sub-sequences by the IEDB tools, and their binding affinity to HLA alleles is also assessed. However, only the peptides verified by PepQuery in Module 3 have evidence supporting their presence in the sample(s) and are outputted in the final report on peptide binders.

The outputs of the pipeline (FASTA-formatted neoantigen peptide candidates and a tabular list of HLA genotypes) can also be used as inputs to the pVACbind software in Galaxy. The user can select from a portfolio of tools in pVACbind for predicting HLA allele binding, and also immunogenicity using deep learning algorithms. The output is a tabular file, which shows all predicted values from each selected algorithm. Users can consult pVACbind documentation to assist in the interpretation of these results [87] and **Supplemental Figure 6(a,b,c)** for guidance on its operation in Galaxy.

This module requires Galaxy-deployed OptiType, Seq2HLA, the IEDB binding prediction software, PepQuery2 (optional), and native Galaxy text processing tools (AWK, Query Tabular). OptiType required 8 CPUs and 32 GB RAM, with runtimes of ~32 hours, while Seq2HLA required 4 CPUs and 16 GB RAM, completing in ~24 hours. The IEDB predictor operated efficiently with 2–4 CPUs and ≤8 GB RAM, processing ~100 peptides in ~30 minutes.

Abbreviations

Browser Extensible Data (BED) formatted file
common Repository of Adventitious Proteins (cRAP)
coding DNA sequence (CDS)
5-aza-2'-deoxycytidine (DAC)
data-dependent acquisition (DDA)
data-independent acquisition (DIA)
eluted ligand (EL)
false-discovery rate (FDR)
Galaxy Training Network (GTN)
Gene Transfer File (GTF)
high-performance computing (HPC)

human leukocyte antigen (HLA)
immunopeptidogenomics (iPepGen)
Insertions and Deletion (InDel)
Integrated Genome Viewer (IGV)
label-free quantification (LFQ)
liquid chromatography (LC)
major histocompatibility complex (MHC)
malignant peripheral nerve sheath tumor (MPNST)
Mascot generic format (.mgf)
mass spectrometry (MS)
National Library of Medicine-National Center for Biotechnology Information (NLM-NCBI)
Next-generation sequencing (NGS)
personalized Variant Antigens in Cancer tool suite (pVACtools)
post-translational modification (PTM)
reads per kilobase per million mapped reads (RPKM)
single amino acid variants (SAVs)
tandem mass spectrometry (MS/MS)
tumor-associated antigen (TAA)
Tumor-specific antigen (TSA)
untranslated region (UTR)

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All the software tools composing the iPepGen pipeline and its workflow modules are available through the Galaxy Tool Shed [111]. The Tool Shed page for each software tool also provides links to GitHub repositories with source code and documentation, along with license information. Most software used in the iPepGen modules is developed using permissive licensing (e.g., GNU GPL, MIT). Some of the tools contained within the FragPipe software platform (MSFragger and the associated IonQuant software are developed under an academic license and are available freely for academic research, non-commercial, or educational

purposes. Other software contained in FragPipe is developed under permissive licensing [112]. The Galaxy bioinformatics platform is freely available and open-source, released under the MIT license [113]. All data, including representative input files, intermediate processed results, and final outputs, are available on the publicly accessible European Galaxy server, as described in **Table 1**. As well as users can also download the data and resultant files from the MassIVE repository MSV000100019 or from PRIDE (PXD071206).

Competing interests

A.I.N. and F.Y. receive royalties from the University of Michigan for the sale of MSFragger, IonQuant, and diaTracer software licenses to commercial entities. All license transactions are managed by the University of Michigan Innovation Partnerships office, and all proceeds are subject to the university's technology transfer policy. D.A.L. is the co-founder and co-owner of NeoClone Biotechnologies, Inc., Discovery Genomics, Inc. (recently acquired by Immusoft, Inc.), B-MoGen Biotechnologies, Inc. (recently acquired by Bio-Techne Corporation), and Luminary Therapeutics, Inc. The business of all these companies is unrelated to the contents of this abstract.

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Authors' contributions

S.M. conceptualized the study, curated data, performed formal analysis, developed software, created visualizations, and drafted and revised the manuscript. R.W. contributed to data curation, software development, and manuscript review. K.T.D. participated in data curation, formal analysis, software development, and manuscript editing. J.E.J. contributed to conceptualization, data curation, and software implementation. F.Y. provided data resources, contributed to software development, and participated in manuscript review. T.J. supported data acquisition and manuscript review. K.R. contributed resources, software support, and manuscript editing. S.C. provided resources and reviewed the manuscript. F.P. assisted in data curation, resource provision, and manuscript editing. A.I.N. contributed software, supervision, funding acquisition, and manuscript review. D.A.L. led conceptualization, resource acquisition,

supervision, funding, and manuscript review. P.D.J. contributed to conceptualization, software development, supervision, funding acquisition, and manuscript review. T.J.G. led conceptualization, software development, supervision, and funding acquisition, and contributed to drafting and revising the manuscript.

All authors read and approved the final manuscript.

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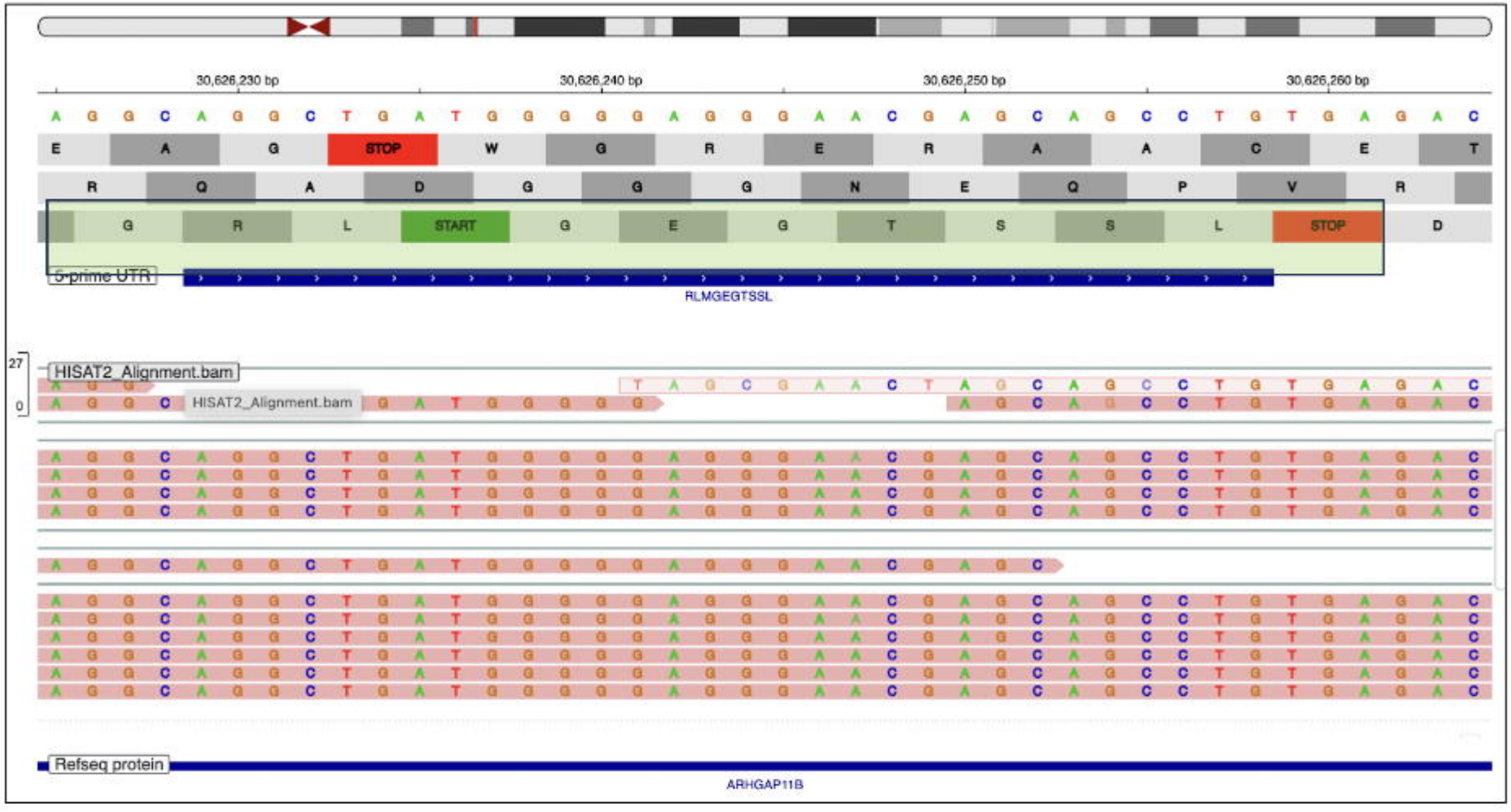
A)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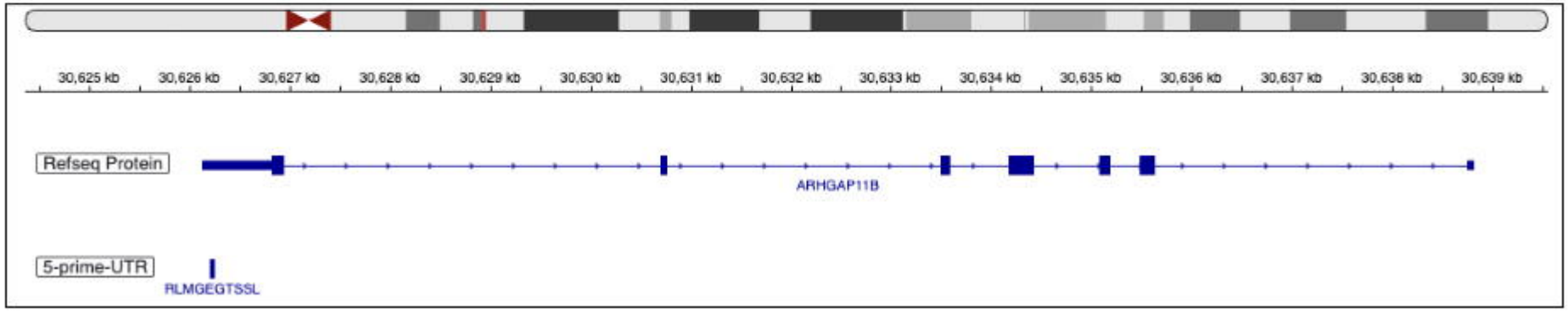
B)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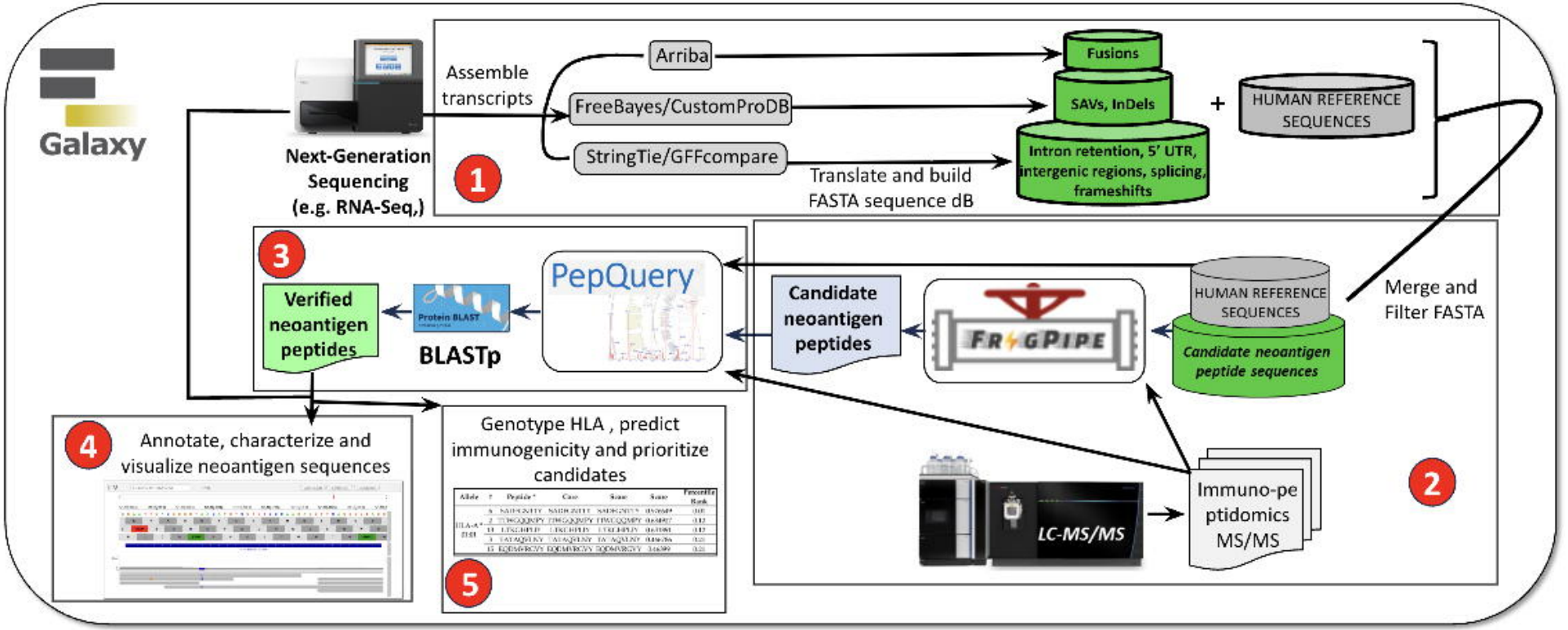
Galaxy
Next-Generation Sequencing (e.g. RNA-Seq,)
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Arriba
FreeBayes/CustomProDB
StringTie/GFFcompare
Translate and build FASTA sequence dB
Fusions
SAVs, InDels
Intron retention, 5' UTR, intergenic regions, splicing, frameshifts
+
HUMAN REFERENCE SEQUENCES
1
3
Verified neoantigen peptides
Protein BLAST
BLASTp
PepQuery
Candidate neoantigen peptides
FRAGPIPE
HUMAN REFERENCE SEQUENCES
Candidate neoantigen peptide sequences
Merge and Filter FASTA
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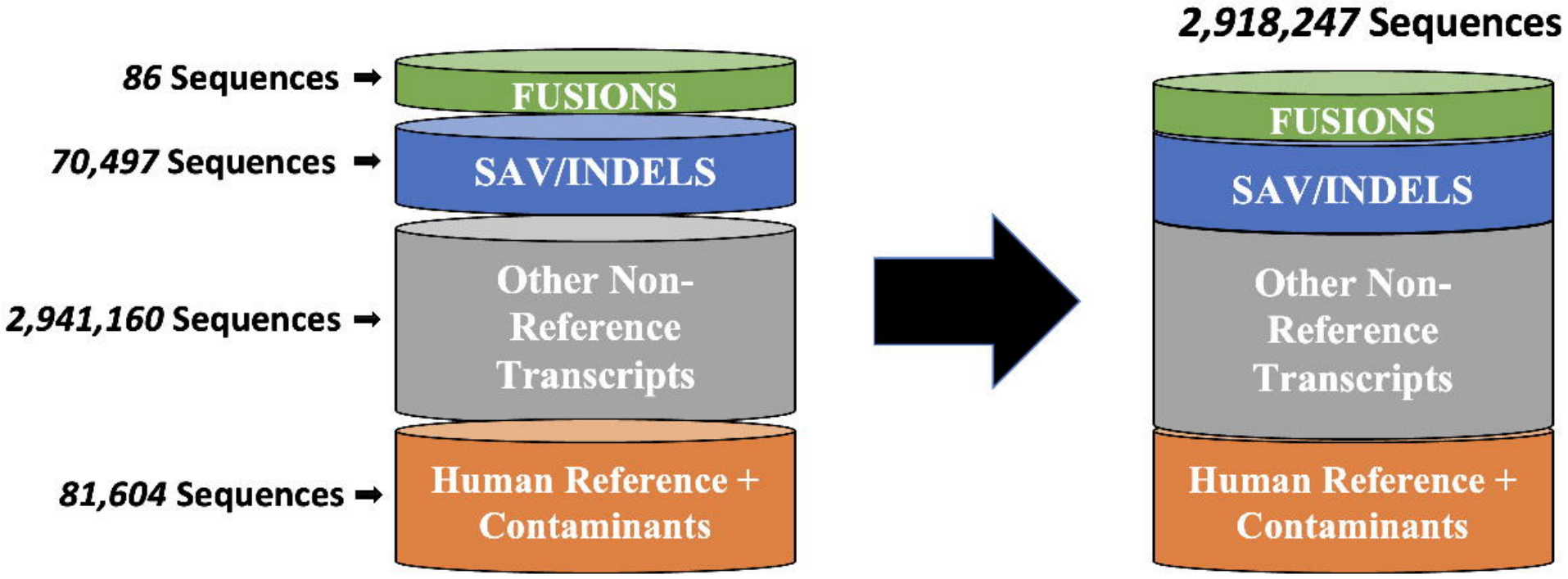
Immuno-peptidomics MS/MS
2
4
Annotate, characterize and visualize neoantigen sequences
Genotype HLA , predict immunogenicity and prioritize candidates
5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SAV/INDELS
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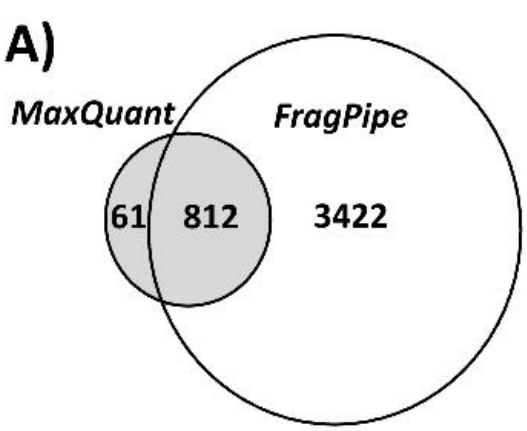
Human Reference + Contaminants


A)
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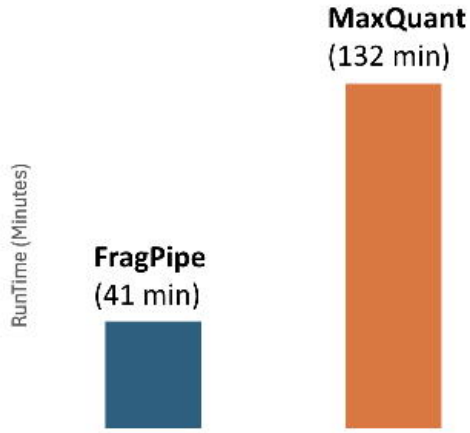
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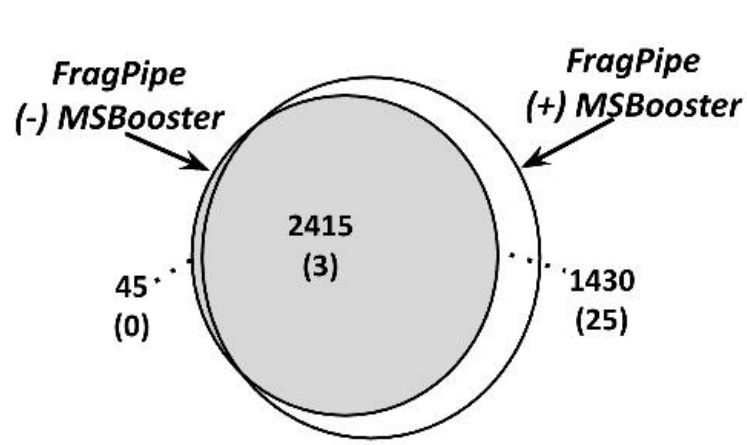
(41 min)
MaxQuant
(132 min)


B)
FragPipe
(-) MSBooster
FragPipe
(+) MSBooster
2415
(3)
45
(0)
1430
(25)