

# 1 Exploring different virulent proteins of human respiratory 2 syncytial virus for designing a novel epitope-based 3 polyvalent vaccine: Immunoinformatics and molecular 4 dynamics approaches

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49 **Abstract**

50 Human Respiratory Syncytial Virus (RSV) is one of the most prominent causes of lower  
51 respiratory tract infections (LRTI), contributory to infecting people from all age groups - a  
52 majority of which comprises infants and children. The implicated severe RSV infections lead  
53 to numerous deaths of multitudes of the overall population, predominantly the children, every  
54 year. Consequently, despite several distinctive efforts to develop a vaccine against the RSV as  
55 a potential countermeasure, there is no approved or licensed vaccine available yet, to control  
56 the RSV infection effectively. Therefore, through the utilization of immunoinformatics tools,  
57 a computational approach was taken in this study, to design and construct a multi-epitope  
58 polyvalent vaccine against the RSV-A and RSV-B strains of the virus. Potential predictions of  
59 the T-cell and B-cell epitopes were followed by extensive tests of antigenicity, allergenicity,  
60 toxicity, conservancy, homology to human proteome, transmembrane topology, and cytokine-  
61 inducing ability. The most promising epitopes (i.e. 13 CTL epitopes, 9 HTL epitopes, and 10  
62 LBL epitopes) exhibiting full conservancy were then selected for designing the peptide fusion  
63 with appropriate linkers, having hBD-3 as the adjuvant. The peptide vaccine was modeled,  
64 refined, and validated to further improve the structural attributes. Following this, molecular  
65 docking analysis with specific TLRs was carried out which revealed excellent interactions and  
66 global binding energies. Additionally, molecular dynamics (MD) simulation was conducted  
67 which ensured the stability of the interactions between vaccine and TLR. Furthermore,  
68 mechanistic approaches to imitate and predict the potential immune response generated by the  
69 administration of vaccines were determined through immune simulations. Owing to an overall  
70 evaluation, *in silico* cloning was carried out in efforts to generate recombinant pETite plasmid  
71 vectors for subsequent mass production of the vaccine peptide, incorporated within *E.coli*.  
72 However, more *in vitro* and *in vivo* experiments can further validate its efficacy against RSV  
73 infections.

75 **Keywords:** Human syncytial respiratory virus; Polyvalent multi-epitope vaccine;  
76 Immunoinformatics; Molecular docking; Molecular dynamics simulation; Immune simulation

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## 93 1. Introduction

94 The Human Respiratory Syncytial Virus (hRSV), a member of the family of *Paramyxoviridae*,  
95 is known to be the primary cause of lower respiratory tract infections (LRTI), including

96 pneumonia and bronchiolitis, in infants, children, as well as elderly and immunocompromised  
97 individuals [1-2]. RSV is an enveloped virus that contains a single-stranded, negative-sense  
98 RNA with a genome size of about 15.2 kb. As of yet, two major RSV antigenic subtypes have  
99 been identified, RSV-A and RSV-B, exhibiting differential sequence divergence throughout  
100 their genome; RSV-A has been seen to be more prevalent than RSV-B [2-3]. Antibody cross-  
101 reactivity patterns revealed these two antigenic subgroups (A and B) for RSV, which were then  
102 divided into genotypes based on genetic divergence within the highly variable G gene [4-6].  
103 Contributory to the fact that the RSV attachment (G) protein has a central conserved domain  
104 (CCD) with a CX3C motif, which has been known to be linked to the generation of protective  
105 antibodies, vaccine candidates including the G protein are of considerable interest [7]. A novel  
106 genotype of RSV-A, known as RSV-A ON1 was found in Ontario, Canada, in 2010. RSV-A  
107 ON1 has a 72-nucleotide duplication at the G Protein's C terminus [8], which has been linked  
108 to an increased risk of pneumonia and lower respiratory tract infections [9]. However, the two  
109 subgroups can coexist and thrive, owing to RSV reinfections being common throughout the  
110 life of an infected individual, indicating that cross-immunity against distinct strains is only  
111 partial [10]. RSV-A infection is commonly followed by RSV-B infection, although the scenario  
112 may vary upon several factors [11]. A claim owing to the antigenic diversity of the G protein  
113 states that, both within and between antigenic subgroups, this prominent diversity aids in  
114 evading pre-existing host immune responses [12, 13].

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117 RSV severely affects immunocompromised infants and the geriatric population with weaned  
118 immune systems. The implicated virus infection is considered globally to be the second largest  
119 cause of death, in children under one year of age. RSV-associated acute LRTI is responsible  
120 for around 33 million serious respiratory infections a year, according to the World Health  
121 Organisation (WHO); resulting in more than 3 million hospitalizations and about 60,000 deaths  
122 of children under 5 years of age, and 6.7% of all deaths in infants younger than one-year-old.  
123 About a half of these hospitalizations and deaths have since been confirmed to be in infants  
124 younger than 6 months of age [14]. Additionally, RSV was identified as the third leading cause  
125 of fatal childhood pneumonia after *Streptococcus pneumonia* and *Haemophilus influenza* in  
126 2005, responsible for approximately 66,000 to 199,000 deaths from pneumonia in children  
127 younger than 5 years [1]. The consequential impact of RSV on older people may be similar to  
128 that of influenza, according to epidemiological research, both in the community and in long-  
129 term care institutions. In nursing facilities, attack rates are around 5–10 percent per year, with  
130 pneumonia (10–20 percent) and mortality (2–5 percent) being quite common. Moreover, RSV  
131 infections cause around 10,000 deaths yearly among those aged 64 and over, according to  
132 estimates based on US healthcare databases and viral surveillance results [15]. RSV infects the  
133 cells lining the human respiration pathway, including the ciliated epithelial cells, and causes  
134 upper and lower respiratory tract complications. Influenza-like diseases and LRTI display  
135 clinical symptoms of serious RSV infection. However, the most frequent and serious  
136 occurrence of infection in younger children is bronchiolitis. Also, over the lifespan of adults,  
137 reinfection by the same and separate strains of RSV is considered to be normal, and therefore,  
138 RSV is often termed as a chronic virus [2].

139  
140 Consequently, over the past two decades, RSV has become a major focus for vaccination  
141 studies to decrease the morbidity of lower respiratory tract infections. Several vaccinations and

142 antiviral drugs have been formulated and implemented over the years since its identification,  
143 and while multiple vaccines, prophylactic and monoclonal antibody candidates are available in  
144 clinical trials, no approved RSV vaccine is currently available to counter RSV [16]. The first  
145 RSV vaccine, composed of the formalin-inactivated virus (FI-RSV) from the Bennett strain,  
146 was studied in a clinical trial back in 1966. Unfortunately, however, the FI-RSV vaccine had a  
147 disastrous effect as it struggled to induce an effective neutralizing antibody response, thus  
148 preventing infection [2]. Large quantities of eosinophils were discovered in the lungs of  
149 children and infants with severe illness, but not in individuals who had a normal RSV infection.  
150 Following this unanticipated outcome, it was crucial to design a safe RSV vaccine, which  
151 included increased testing for vaccine-induced illnesses [17-21]. The inability of the vaccine  
152 to elicit effective neutralizing antibodies or memory CD8+ T cells, as well as the production  
153 of a significant inflammatory CD4 T cell response, contributed to this vaccine-induced illness  
154 [22-25].

155 Numerous modified RSV vaccine candidates have been designed after the failure of the FI-  
156 RSV vaccine trial and many of them are now in clinical trials. However, none of those vaccine  
157 candidates being licensed have so far made it to the international economy, for mass production  
158 and administration. Although live-attenuated vaccines can stimulate both a humoral and  
159 cellular immune response, clinical trials have revealed some potential drawbacks.  
160 Chimpanzees are used to compare the amount of attenuation of vaccinations that are candidates  
161 for use in humans. Karron et al. found that RSV vaccines that were temperature sensitive and  
162 had a high degree of attenuation in chimps could cause infection in the lower respiratory tract  
163 in children [26]. Furthermore, recombinant vector-based vaccinations allow for the  
164 presentation of one or more antigens encoded on a viral vector such as PIV3 or adenovirus  
165 [27]. Intranasal delivery of a new BLP (bacterial-like particle) conjugated to the RSV fusion  
166 protein stimulates both mucosal IgA responses and increased IFN-production in a different sort

167 of vaccination approach [28]. Although both represent effective approaches, further assays to  
168 evaluate the long-lasting immune responses are paramount [29].

169 In addition, it has been observed that, with the use of RSV vaccine candidates, palliative  
170 treatment with RSV anti-infective drugs is also required [30]. Merely two approved RSV  
171 antivirals are currently available, which include, palivizumab, a humanized preventive  
172 monoclonal antibody, and aerosolized ribavirin for therapy. The symptoms of RSV infections  
173 can be alleviated by these two antivirals, although they cannot serve prophylactic measures  
174 [31]. While studies are underway to identify an effective antiviral therapy or countermeasure  
175 to prevent RSV spread and infection, these studies have not been able to deliver any satisfactory  
176 findings that can be used to tackle RSV infections [32].

177 The production of a viable vaccine candidate against a specific pathogen by traditional means  
178 can often take many years [33]. However, the age of vaccine production, especially the novel  
179 epitope-based "subunit vaccines," has been enriched by today's modern technology and the  
180 availability of genomic information for almost all pathogens. These subunit vaccines consist  
181 only of the antigenic protein segments of the target pathogen and hence, toxic and  
182 immunogenic or allergenic parts of the antigen can be dissipated during the construction of the  
183 specific vaccine [34]. Again, the development of vaccines using these computer-based  
184 approaches takes far less time, and thus greatly reduces the expense of construction and  
185 development [35, 36].

186 The immunoinformatics approach in this study was used to establish successful polyvalent  
187 vaccines against the virulent strains of both forms of RSV, i.e. RSV-A and RSV-B respectively.  
188 Immunoinformatics is a vaccine modeling process that allows predictions using several  
189 computational methods. The novel antigens of a pathogen or virus are identified in  
190 immunoinformatics by dissecting its genomic data and then, through the utilization of various  
191 *in silico* biology and bioinformatics tools for vaccine design and development, by analyzing

192 the target pathogen genome [35, 37]. In our research, a polyvalent epitope-based vaccine  
193 blueprint was produced that could produce a significant immune response to both RSV-A and  
194 RSV-B forms, targeting the phosphoprotein (P protein), nucleoprotein (N protein), fusion  
195 glycoprotein (F protein), and major surface glycoprotein (mG protein) of these viruses. Since  
196 RSV-A is more prevalent than RSV-B, as a model, the vaccine was developed using RSV-A  
197 [2]. For the T-cell and B-cell epitope prediction, the RSV-A P protein, N protein, F protein,  
198 and mG protein were used and then the epitopes with 100 % conservancy in both species along  
199 with some other selection criteria were selected for vaccine construction. The criteria for  
200 selecting the epitopes include i.e., antigenicity (the parameter that measures whether the  
201 epitopes stimulate a high antigenic response), non-allergenicity (to ensure that the epitopes do  
202 not cause any unintended allergic reaction inside the body), non-toxicity, conservancy across  
203 the selected organisms, as well as non-homologation of the human proteome. It is, therefore,  
204 expected that the vaccine will be effective against both the subtypes - RSV-A and RSV-B. The  
205 most common vaccine target for RSV is known to be the F protein [574 amino acids (aa) in  
206 length], which is a highly conserved protein in both RSV forms. The F protein mediates the  
207 fusion and attachment of the virus to its target cells along with the mG protein, thus facilitating  
208 viral entry [2, 38]. The F1 (aa 137–574) and F2 (aa 1–109) subunits form a homotrimer in the  
209 mature F protein, and the F1 subunit is required for membrane fusion. The F protein has two  
210 different conformations i.e., the pre-fusion and post-fusion conformations [39, 40]. The protein  
211 rearranges to a more stable post-fusion form during infection to allow viral entrance into the  
212 host cell. Antibodies having neutralizing activity identify at least two antigenic sites on both  
213 the pre-fusion and post-fusion forms of F (sites II and IV) [41-43]. In this study, the precursor  
214 F0 protein was targeted to retrieve all of the potential antigenic epitopes. The possible  
215 conformational change of the F protein, as well as the cleavage sites of the protein sequence,  
216 were taken into account while generating the potential epitopes [39, 40].

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218 The viral genome of RSV is surrounded by N protein, and the P protein is a vital component  
219 of the viral RNA-dependent RNA polymerase complex which is necessary for the proper  
220 replication and transcription of RSV [44]. Therefore, in our study, these four proteins were  
221 used as possible targets to design a vaccine to suppress these viral proteins, preventing viral  
222 entry, and thus interfering with the life cycle of the virus.

223

## 224 **2. Methods and Materials**

225 The high throughput immunoinformatics and MD approaches of vaccine designing are  
226 illustrated in a step-by-step processes in **Fig 1**.

227

228 **Fig 1.** The step-by-step procedures of immunoinformatics and molecular dynamics approaches  
229 used in the vaccine designing study.

### 230 **2.1. Protein sequences identification and retrieval**

231 Through existing literature reviews in the National Center for Biotechnology Information  
232 (NCBI) (<https://www.ncbi.nlm.nih.gov/>) database, the RSV-A and RSV-B viruses were  
233 identified and selected along with their target proteins (i.e., P protein, N protein, F protein, and  
234 mG protein). The sequences of target proteins of the selected strains (i.e., RSV strain A2 and  
235 RSV strain B1) were then extracted from the UniProt (<https://www.uniprot.org/>) database in  
236 FASTA format. The NCBI Protein database is a collection of SwissProt, PIR, PRF, and PDB  
237 sequences. It also includes GenBank, RefSeq, and TPA translations from elucidated coding  
238 regions.

239 **2.2. Prediction of antigenicity and analysis of physicochemical**  
240 **properties of the selected proteins**

241 Using the online antigenicity prediction tool, VaxiJen v2.0 (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>), the antigenicity of the target protein sequences  
242 was predicted with the prediction precision parameter threshold kept at 0.4. This tool uses the  
243 method of transformation of auto cross-covariance (ACC) to predict the antigenicity of query  
244 proteins or peptides and provides results with an accuracy of 70% to 89%. For this reason, this  
245 server is the widely used and accepted server to determine the antigenicity of query proteins  
246 [45]. ProtParam tool of the ExPASy server (<https://web.expasy.org/protparam/>) has  
247 subsequently determined numerous physicochemical properties, i.e. the number of amino  
248 acids, molecular weight, number of total atoms, theoretical pI, instability index, extinction  
249 coefficient, half-life, grand average of hydropathicity (GRAVY), etc. of the target proteins  
250 [46].

252 **2.3. Prediction of T-cell and B-cell epitopes**

253 The two major types of T-cells, cytotoxic T-cells, and Helper T-cells are both considered  
254 essential for the successful design of the vaccine [47]. For specific antigen recognition of the  
255 major histocompatibility complex class I (MHC-I) or CD8+ cytotoxic T-lymphocytic (CTL)  
256 epitopes on the surface of the antigen-presenting cells (APCs), the cytotoxic T-cells are  
257 important. Additionally, the helper T-cells are considered to be a crucial component of adaptive  
258 immunity that interacts on the surface of APCs with major histocompatibility complex class II  
259 (MHC-II) or CD4+ helper T-lymphocytic (HTL) epitopes. They function in activating the B-  
260 cell, macrophages, and even cytotoxic T-cells [48, 49]. On the other hand, B-cells produce  
261 antigen-specific immunoglobulins after their activation [50]. They can identify solvent-

262 exposed antigens via membrane-bound immunoglobulins called B cell receptors (BCRs) [51].  
263 B-cell epitopes are important for defense against viral infections because they are the essential  
264 immune system components that activate an adaptive immune response in response to a  
265 specific viral infection. Therefore, the B-cell epitopes are used as one of the crucial building  
266 blocks of the subunit vaccine. There are two types of B-cell epitopes: linear B-cell epitopes  
267 (LBL) and conformational B-cell epitopes, also known as continuous and discontinuous B-cell  
268 epitopes, respectively [52].

269 The T-cell and B-cell epitope prediction was performed using the Immune Epitope Database  
270 or IEDB (<https://www.iedb.org/>), which contains extensive experimental data on antibodies  
271 and epitopes [53]. For the prediction of MHC Class-I or CTL epitopes for several human  
272 leukocyte antigen (HLA) alleles, i.e., HLA A\*01:01, HLA A\*03:01, HLA A\*11:01, HLA  
273 A\*02:01, HLA A\*02:06, and HLA A\*29:02, the recommended IEDB NetMHCpan 4.0  
274 prediction method was used. The default prediction method selection of the server is 'IEDB  
275 recommended' which utilizes the best available technique for a specific MHC molecule based  
276 on the availability of predictors and observes the predicted performance for a specific allele. It  
277 is updated regularly based on predictor availability. NetMHCpan EL 4.1 is currently used  
278 across all alleles for peptide: MHC Class-I binding prediction. Again, for the prediction of  
279 MHC class-II or HTL epitopes for DRB1\*03:01, DRB1\*04:01, DRB1\*15:01, DRB3\*01:01,  
280 DRB5\*01:01, and DRB4\*01:01 alleles, the recommended IEDB 2.22 prediction method was  
281 used. If any corresponding predictor is available for the MHC molecule, the IEDB  
282 recommended method employs the Consensus method, combining NN-align, SMM-align,  
283 CombLib, and Sturniolo; otherwise, NetMHCIIPan is used. If any three of the four approaches  
284 are available, the Consensus approach evaluates them all, with Sturniolo as the final option.  
285 Henceforth, based on their ranking, the top-scored HTL and CTL epitopes that were found to  
286 be common for all of the selected corresponding HLA alleles were considered for further

287 analyses. All the parameters were retained by opting for default during the T-cell epitope  
288 prediction. Subsequently, B-cell epitopes of the proteins were predicted using the BepiPred  
289 linear epitope prediction method 2.0, maintaining all the default parameters. Using a Random  
290 Forest algorithm trained on epitope and non-epitope amino acids obtained from crystal  
291 structures, the BepiPred-2.0 server predicted linear B-cell epitopes from a protein sequence.  
292 Following this, a sequential prediction smoothing was conducted. Residues with scores greater  
293 than the threshold (default value of 0.5) were thought to constitute epitopes [54]. Finally, the  
294 top-scored LBL epitopes containing more than ten amino acids were primarily regarded as  
295 potential candidates for further analysis.

296 Conformational or discontinuous B-cell epitopes are critical components to induce antibody-  
297 mediated humoral immunity within the body. While designing a vaccine, efficient  
298 conformational B-cell epitopes should be included alongside the LBLs to elicit a better  
299 immunogenic response against the pathogen. The conformational B-cell epitopes of the  
300 modeled 3D structure of the vaccine were predicted using IEDB ElliPro, an online server  
301 (<http://tools.iedb.org/ellipro/>) using the default parameters of a minimum score of 0.5 and a  
302 maximum distance of 6 angstroms [55]. ElliPro uses three algorithms to predict the protein  
303 shape as an ellipsoid, measure the residue PI, and estimate adjacent cluster residues based on  
304 their protrusion index (PI) values [56]. ElliPro calculates a score for each output epitope based  
305 on an average PI value over the residues of each epitope. Protein residues are contained in 90%  
306 of ellipsoids with a PI value of 0.9, while 10% of residues are outside ellipsoids. The center of  
307 residue mass residing outside the largest ellipsoid possible was used to calculate the PI value  
308 for each epitope residue [57].

309 **2.4. Assessment of antigenicity, allergenicity, toxicity, and topology**  
310 **prediction of the epitopes**

311 In this step, several methods for predicting their conservancy, antigenicity, allergenicity, and  
312 toxicity were used to evaluate the initially predicted T-cell and B-cell epitopes. To assess the  
313 conservancy of the chosen epitopes [58], the conservancy prediction method of the IEDB  
314 server (<https://www.iedb.org/conservancy/>) was used. Additionally, the components of the  
315 vaccine should be highly antigenic, non-allergenic at the same time, and also devoid of toxic  
316 reactions. In this step, the antigenicity determination tool VaxiJen v2.0 (<http://www.ddg->  
317 [pharmfac.net/vaxijen/VaxiJen/VaxiJen.html](http://pharmfac.net/vaxijen/VaxiJen/VaxiJen.html)) was used again for the determination of  
318 antigenicity [45]. Two different tools were then used, i.e. AllerTOP v2.0 (<https://www.ddg->  
319 [pharmfac.net/AllerTOP/](http://pharmfac.net/AllerTOP/)) and AllergenFP v1.0 (<http://ddg-pharmfac.net/AllergenFP/>) to obtain  
320 the highest precision for prediction of allergenicity. Both of the tools are based on auto cross-  
321 covariance (ACC) transformation of protein sequences into uniform equal-length vectors.  
322 However, the AllerTOP v2.0 server has a better 88.7 % prediction accuracy than the  
323 AllergenFP v1.0 server (87.9 %) [59, 60]. In addition, the ToxinPred  
324 (<http://crdd.osdd.net/raghava/toxinpred/>) server was used to predict toxicity for all epitopes by  
325 using the Support Vector Machine (SVM) prediction method to keep all the default parameters.  
326 The SVM is a widely accepted machine learning technique for toxicity prediction since it can  
327 differentiate the toxic and non-toxic epitopes quite efficiently [61]. Finally, using the TMHMM  
328 v2.0 server (<http://www.cbs.dtu.dk/services/TMHMM/>), the transmembrane topology  
329 prediction of all the epitopes was performed to predict whether the epitopes were exposed  
330 inside or outside, keeping the parameters at their default values. TMHMM uses an algorithm  
331 called N-best (or 1-best in this case) to predict the most probable location and orientation of  
332 transmembrane helices in the sequence [62].

333 **2.5. Cytokine inducing capacity prediction of the epitopes**

334 Several cytokine types, including IFN- $\gamma$ , IL-4 (interleukin-4), and IL-10 (interleukin-10) are  
335 produced by helper T cells to activate various immune cells, i.e. cytotoxic T cells,  
336 macrophages, etc. [63]. As a result, it is crucial to know whether HTL epitopes are capable of  
337 producing key cytokines to induce an immune response against the virus before designing a  
338 vaccine. The induction capacity of the predicted HTL epitopes for interferon- $\gamma$  (IFN- $\gamma$ ) was  
339 determined using the IFNepitope (<http://crdd.osdd.net/raghava/ifnepitope/>) server. Based on  
340 analyzing a dataset that includes IFN- $\gamma$  inducing and non-inducing peptides, the server  
341 determines the probable IFN- $\gamma$  inducing epitopes. To determine the IFN- $\gamma$  inducing capacity,  
342 the Design module and the Hybrid (Motif + SVM) prediction approach were used. The Hybrid  
343 prediction approach is considered to be a highly precise approach to the prediction of the  
344 epitope-inducing capacity of IFN- $\gamma$  [64]. In addition, IL-4 and IL-10 inducing HTL epitope  
345 properties were determined using the servers IL4pred  
346 (<https://webs.iiitd.edu.in/raghava/il4pred/index.php>) and IL10pred  
347 (<http://crdd.osdd.net/raghava/IL-10pred/>) [65, 66]. The SVM method was used on both servers,  
348 where the default threshold values were kept at 0.2 and -0.3, respectively.

## 349 **2.6. Conservancy and human proteome homology prediction**

350 The conservancy analysis of the specified epitopes was conducted using the IEDB server's  
351 epitope conservancy analysis module (<https://www.iedb.org/conservancy/>) [58]. The epitopes  
352 that were found to be fully conserved among the selected strains were taken for the construction  
353 of the vaccine since this will ensure and facilitate the broad-spectrum activity of the polyvalent  
354 vaccine over the two selected RSV species or types. The homology of the human proteome  
355 epitopes was determined by the BLAST (BlastP) protein module of the BLAST tool  
356 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), where Homo sapiens (taxid:9606) was used for  
357 comparison, keeping all other default parameters. An e-value cut-off of 0.05 was set and

358 epitopes were selected as non-homologous pathogen peptides that showed no hits below the e-  
359 value inclusion threshold [67]. The epitopes found to be highly antigenic, non-allergenic, non-  
360 toxic, fully conserved, and non-homologous to the human proteome were considered among  
361 all the initially selected epitopes to be the best-selected epitopes or the most promising epitopes,  
362 and only these selected epitopes were used in the construction of the vaccine.

363 **2.7. Population coverage and cluster analyses of the epitopes and**  
364 **their MHC alleles**

365 A crucial requirement is to consider the distribution of unique HLA alleles among the different  
366 populations and ethnicities around the world to design a multi-epitope vaccine since the  
367 expression of different HLA alleles can vary from population to population. The IEDB resource  
368 for population coverage (<http://tools.iedb.org/population/>) was used for analyzing the  
369 population coverage of the most promising epitopes across several HLA alleles in various  
370 regions around the world. Denominated MHC restriction of T cell responses and polymorphic  
371 HLA combinations were considered in the analysis. All the parameters were maintained at their  
372 default conditions during the study.

373 Furthermore, the human MHC genomic region or HLA is enormously polymorphic, with  
374 thousands of alleles; many of which code for a different molecule. MHCcluster is a program  
375 that organizes the MHC molecules into functional clusters based on their predicted binding  
376 specificity. The approach provides a user-friendly online interface that allows the user to  
377 include any MHC in the analysis. A static heat map and graphical tree-based visualizations of  
378 the functional relationship between the MHC variants are included in the output as well as a  
379 dynamic TreeViewer interface that displays both the functional relationship and the individual  
380 binding specificities of the MHC molecules [68]. To evaluate the relationship between the  
381 selected MHC alleles, cluster analysis of the MHC alleles was done using the online tool

382 MHCcluster 2.0 <https://services.healthtech.dtu.dk/service.php?MHCcluster-2.0>). During the  
383 study, 50,000 peptides to be used were retained, 100 bootstrap measurements were retained,  
384 and both HLA super-type (MHC Class-I) and HLA-DR (MHC class-II) members were chosen.

## 385 **2.8. Designing of the multi-epitope subunit vaccine**

386 The most promising antigenic epitopes have been linked with each other to create a fusion  
387 peptide using an adjuvant and linkers. Human beta-defensin-3 (hBD-3) used an adjuvant  
388 sequence that was linked to the epitopes by EAAAK linkers. Adjuvants are considered to play  
389 important roles in improving the antigenicity, immunogenicity, stability, and durability of the  
390 developed vaccine. The hBD-3 plays a vital role in host immune responses against the  
391 pathogens (i.e., innate mucosal defense within the respiratory tract) and is highly significant  
392 against respiratory infections [69-71].

393 The epitopes were also appended to the pan HLA-DR epitope (PADRE) sequence. By  
394 enhancing the ability of CTL vaccine epitopes, the PADRE sequence activates the immune  
395 responses [34]. In the conjugation of the CTL, HTL, and LBL epitopes, the AAY, GPGPG,  
396 and KK linkers were used, respectively. The EAAAK linkers have a viable partition of  
397 bifunctional fusion protein domains [72], while the GPGPG linkers are ideal for preventing  
398 junctional epitope production and optimizing the processing and presentation of the immune  
399 system [73]. The AAY linker is also commonly used in the design trials of the *in silico* vaccine  
400 since this linker offers successful and efficient epitope conjugation [74]. In addition, bi-lysine  
401 (KK) linkers are active in the autonomous immunological function of vaccine epitopes [75].

## 402 **2.9. Physicochemical property analyses of the vaccine with 403 antigenicity and allergenicity test**

404 To build a timely and successful immune response to the pathogenic attack, the constructed  
405 vaccine should be strongly antigenic. The antigenicity of the vaccine model was estimated  
406 using VaxiJen v2.0 (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.htm>), keeping the  
407 threshold value fixed at 0.4 [45]. The findings of the Vaxijen v2.0 server were further cross-  
408 checked by the ANTIGENpro module of the SCRATCH protein predictor  
409 (<http://scratch.proteomics.ics.uci.edu/>), holding all the default parameters [76], to achieve  
410 better prediction precision. Three separate online methods have estimated the allergenicity of  
411 the vaccine structures, i.e. AlgPred (<http://crdd.osdd.net/raghava/algpred/>), AllerTop v2.0  
412 (<https://www.ddgpharmfac.net/AllerTOP/>) and AllergenFP v1.0 ([dg-](http://dg-)  
413 <http://www.ddgpharmfac.net/AllergenFP/>), to ensure optimum prediction precision. The AlgPred  
414 (<http://crdd.osdd.net/raghava/algpred/>) server aims to combine multiple allergenicity  
415 determination methods to reliably determine possible allergenic proteins [77, 78]. To predict  
416 the vaccine's allergenicity, the MEME/MAST motif prediction approach was used. The  
417 physicochemical properties of the built vaccine were then estimated by the same online  
418 instrument, ProtParam (<https://web.expasy.org/protparam/>)[15], which was previously  
419 utilized. The solubility of vaccine constructs was also estimated alongside the physicochemical  
420 property study by the SOLpro module of the SCRATCH protein predictor  
421 (<http://scratch.proteomics.ics.uci.edu/>) and later further explained by the Protein-Sol server  
422 (<https://protein-sol.manchester.ac.uk/>). The solubility of a query protein is predicted by all  
423 these servers with remarkable precision. SolPro produces its predictions based on the SVM  
424 method, while Protein-Sol uses a rapid method of deciding the results based on sequence [76,  
425 79]. All the parameters of the servers were maintained at their default values during the  
426 solubility review.

427 **2.10. Secondary and tertiary structure prediction of the vaccine  
428 construct**

429 The vaccine construct was subjected to secondary structure prediction following  
430 physicochemical analysis. For this, several online resources were used to preserve all the  
431 default parameters, i.e. PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred/>) (using the PSIPRED 4.0  
432 prediction method), GOR IV ([https://npsa-prabi.ibcp.fr/cgibin/npsa\\_automat.pl?page=/NPSA/npsa\\_gor4.html](https://npsa-prabi.ibcp.fr/cgibin/npsa_automat.pl?page=/NPSA/npsa_gor4.html)), SOPMA ([https://npsa-prabi.ibcp.fr/cgibin/npsa\\_automat.pl?page=/NPSA/npsa\\_sopma.html](https://npsa-prabi.ibcp.fr/cgibin/npsa_automat.pl?page=/NPSA/npsa_sopma.html)) and SIMPA96  
433 ([https://npsa-prabi.ibcp.fr/cgibin/npsa\\_automat.pl?page=/NPSA/npsa\\_npsa.html](https://npsa-prabi.ibcp.fr/cgibin/npsa_automat.pl?page=/NPSA/npsa_npsa.html)) and SIMPA96  
434 (<https://npsa.npsa.npsa.npsa>). To predict the percentages or quantities of amino acids in  $\alpha$   
435 helix,  $\beta$ -sheet, and coil structure formations, these servers are considered to be reliable, quick,  
436 and effective [80-84]. Moreover, determination of the tertiary or 3D structure of the vaccine  
437 construct was carried out using the RaptorX online server (<http://raptordb.uchicago.edu/>). Using  
438 an easy and powerful template-based method [85], the server predicts the tertiary or 3D  
439 structure of a query protein. Furthermore, RaptorX uses a deep learning method to enable  
440 distance-based protein folding. This server has also been rated first in contact prediction in both  
441 CASP12 and CASP13, making it an ideal server for 3D structure determination [86].

#### 444 **2.11. Refinement and validation of tertiary structure of the vaccine**

445 The tertiary structure prediction of the proteins using computational methods also requires  
446 extensive refinement, to turn predicted models with lower resolution into models that closely  
447 match the native protein structure. Therefore, a GalaxyWEB server (<http://galaxy.seoklab.org/>)  
448 using the GalaxyRefine module further refined the created tertiary structure of the proposed  
449 vaccine model. The server uses dynamic simulation and the refinement approach is tested by  
450 CASP10 to refine the tertiary protein structures [87, 88]. Furthermore, validation of the refined  
451 protein was carried out by analyzing the Ramachandran plot created by the PROCHECK  
452 (<https://servicesn.mbi.ucla.edu/PROCHECK/>) tool [89, 90]. Along with PROCHECK for  
453 protein validation, another online platform, ProSA-web

454 (https://prosa.services.came.sbg.ac.at/prosa.php) was also used. A z-score that expresses the  
455 consistency of a query protein structure is created by the PROCHECK server. In the latest PDB  
456 database, a z-score residing within the z-score spectrum of all experimentally defined protein  
457 chains represents a higher consistency of the query protein [91].

458 **2.12. Vaccine protein disulfide engineering analysis**

459 Disulfide bonds are more likely to form in a few regions within a protein structure, providing  
460 stability through reduced conformational entropy and increased free energy concerning the  
461 denatured state. However, disulfide engineering is the process of introducing disulfide bonds  
462 to a target protein to increase its stability. In this experiment, the Disulfide by Design (DbD)2  
463 v12.2 (<http://cptweb.cpt.wayne.edu/DbD2/>) online tool was used to predict the locations and  
464 further design the disulfide bonds within the vaccine proteins [92]. The tool was developed  
465 using computational approaches to predict the protein structure [93, 94], and the algorithm of  
466 this server accurately estimates the  $\chi_3$  torsion angle based on the C $\beta$ –C $\beta$  distance using a  
467 geometric model derived from native disulfide bonds. The Caf-C $\beta$ -S $\gamma$  angle is allowed some  
468 tolerance in the DbD2 server based on the wide range found in native disulfides. To facilitate  
469 the ranking process, DbD2 estimates an energy value for each potential disulfide and mutant  
470 PDB files may be generated for selected disulfides [95].

471 The  $\chi_3$  angle was held at -87 ° or +97 °  $\pm$ 10 during the experiment to cast off various putative  
472 disulfides that were generated using the default angles of +97 °  $\pm$ 30 ° and -87 °  $\pm$ 30 °.  
473 Additionally, the angle of Caf-C $\beta$ -S $\gamma$  was set to its default value of 114.6°  $\pm$ 10. Finally, to  
474 allow disulfide bridge formation, residue pairs with energy less than 2.2 Kcal/mol were selected  
475 and mutated to cysteine residue [96]. The energy value of 2.2 Kcal/mol was chosen as the  
476 disulfide bond selection threshold since 90% of native disulfide bonds are usually considered  
477 to have an energy value of less than 2.2 Kcal/mol [92].

## 478 **2.13. Post-translational modification analysis**

479 For posttranslational modification analysis of the vaccine construct comprising of the B-cell  
480 and T-cell epitopes, the NetNGlyc-1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc-1.0>),  
481 NetOGlyc4.0 (<http://www.cbs.dtu.dk/services/NetOGlyc-4.0>), and NetPhos-3.1  
482 (<http://www.cbs.dtu.dk/services/NetPhos-3.1>) servers were utilized. The NetNglyc server uses  
483 artificial neural networks to predict N-glycosylation sites in human proteins by examining the  
484 sequence context of Asn-Xaa-Ser/Thr sequons [97]. Any potential that exceeds the default  
485 threshold of 0.5 indicates a predicted glycosylated site. The average output of nine neural  
486 networks is used to get the 'potential' score. The NetOglyc server  
487 (<http://www.cbs.dtu.dk/services/NetOGlyc-4.0>) predicts mucin type GalNAc O-glycosylation  
488 sites in mammalian proteins using neural networks [98].  
489 This server provides a list of probable glycosylation sites for each input sequence, together  
490 with their positions in the sequence and prediction confidence scores. Only locations with a  
491 score greater than 0.5 are expected to be glycosylated and the string "POSITIVE" is added to  
492 the remark box. Using ensembles of neural networks, the NetPhos 3.1 server  
493 (<http://www.cbs.dtu.dk/services/NetPhos-3.1>) predicts serine, threonine, or tyrosine  
494 phosphorylation sites in eukaryotic proteins. Predictions are made for both generic and kinase-  
495 specific kinases. A prediction score greater than 0.5 indicates a positive prediction.

## 496 **2.14. Analysis of protein-protein docking**

497 The vaccine protein was docked against several toll-like receptors (TLRs) in protein-protein  
498 docking analysis. A strong binding affinity should be present between the vaccine and the  
499 TLRs. This is crucial because, after identifying the vaccine that resembles the initial viral  
500 infections, TLR proteins generate possible immune responses, and thus help to produce  
501 immunity against the pathogen [99]. In this study, different TLRs have been docked with the

502 vaccine protein, i.e. TLR-1 (PDB ID: 6NIH), TLR-2 (PDB ID: 3A7C), TLR-3 (PDB ID:  
503 2A0Z), TLR-4 (PDB ID: 4G8A), and TLR9 (PDB ID: 3WPF). ClusPro v2.0  
504 (<https://cluspro.bu.edu/login.php>) was used to conduct the docking, where the lower energy  
505 score corresponds to the stronger binding affinity. Based on the following equation, the ClusPro  
506 server calculates the energy score:

507 
$$E = 0.40E_{rep} + (-0.40E_{att}) + 600E_{elec} + 1.00E_{DARS} [54 - 55].$$

508 The repulsions and attraction energies owing to van der Waals interactions are denoted by  $E_{rep}$   
509 and  $E_{attr}$ , respectively, whereas  $E_{elec}$  signifies the electrostatic energy component. The Decoys'  
510 pairwise structure-based potential is represented by  $E_{DARS}$  as the Reference State (DARS)  
511 method. Furthermore, another round of docking was carried out using the ZDOCK server  
512 which is a rigid-body protein-protein docking tool that employs a combination of shape  
513 complementarity, electrostatics, and statistical potential terms for scoring and uses the Fast  
514 Fourier Transform algorithm to enable an efficient global docking search on a 3D grid. In the  
515 most current benchmark version (Accelerating protein docking in ZDOCK utilizing an  
516 advanced 3D convolution library), ZDOCK achieves high predictive accuracy on protein-  
517 protein docking benchmarks, with >70 % success in the top 1000 predictions for rigid-body  
518 instances [100].

519 **2.15. Molecular dynamics simulation studies and MM-PBSA**  
520 **calculations**

521 The docked complexes from the ZDOCK server were used in MD simulations. The complexes  
522 being protein-protein in nature with multiple chains, the MD simulations were computationally  
523 expensive and performed on the HPC cluster at Bioinformatics Resources and Applications  
524 Facility (BRAF), C-DAC, Pune with Gromacs 2020.4 [101] MD simulation package. The  
525 CHARMM-36 force field parameters [102, 103] were employed to prepare the topology of

526 protein chains. The system of each TLR along with the bound vaccine was solvated with the  
527 single point charge water model [104] in the dodecahedron unit cells and neutralized with the  
528 addition of  $\text{Na}^+$  or  $\text{Cl}^-$  counter-ions. The solvated systems were initially energy minimized to  
529 relieve the steric clashes if any with the steepest descent criteria until the threshold ( $\text{Fmax} < 10$   
530  $\text{kJ/mol}$ ) was reached. These energy minimized systems were then equilibrated at constant  
531 volume and temperature conditions 300 K using modified Berendsen thermostat [105] and then  
532 at constant volume and pressure Berendsen barostat [106] for 100 ps each. The equilibrated  
533 systems were later subjected to 100 ns production phase MD simulations, where the modified  
534 Berendsen thermostat and Parrinello-Rahman barostat [107] were used with covalent bonds  
535 restrained using the LINCS algorithm [108]. The long-range electrostatic interaction energies  
536 were measured with the cut-off of 12 Å, with the Particle Mesh Ewald method (PME) [109].  
537 The resulting trajectories were analyzed for root mean square deviations (RMSD) in protein  
538 backbone atoms, root mean square fluctuations (RMSF) in the side chain atoms of individual  
539 chains in each protein complex, the radius of gyration ( $\text{Rg}$ ), and several hydrogen bonds formed  
540 between vaccine protein chain and the respective TLR protein chain.

## 541 **2.16. Immune simulation studies**

542 To forecast the immunogenicity and immune response profile of the proposed vaccine, an  
543 immune simulation analysis was performed. For the immune simulation study, the C-ImmSim  
544 server (<http://150.146.2.1/CIMMSIM/index.php>) was used to predict real-life immune  
545 interactions using machine learning techniques and PSSM (Position-Specific Scoring Matrix)  
546 [110]. During the experiment, all the variables except for the time steps were kept at their  
547 default parameters. However, the time steps at 1, 84, and 170 were retained (time step 1 is  
548 injection at time = 0), and the number of simulation steps was set to 1050. Thus, three injections  
549 at four-week intervals were administered to induce recurrent antigen exposure [111].

## 550 **2.17. Codon adaptation and *in silico* cloning within *E.coli* System**

551 Codon adaptation and *in silico* cloning are two significant steps that are conducted to express  
552 multi-epitope vaccine construction within an *Escherichia coli* (*E.coli*) K12 strain. In different  
553 organisms, an amino acid can be encoded by more than one codon, which is known as codon  
554 bias wherefore, the codon adaptation study is carried out to predict an appropriate codon that  
555 effectively encodes a specific amino acid in a specific organism. Java Codon Adaptation Tool  
556 or JCat server (<http://www.jcat.de/>) was used for codon optimization [112], and the optimized  
557 codon sequence was further analyzed for expression parameters, codon adaptation index (CAI),  
558 and GC-content %. The optimum CAI value is 1.0, while a score of > 0.8 is considered  
559 acceptable, and the optimum GC content ranges from 30 to 70% [113]. For *in silico* cloning  
560 simulation, the pETite vector plasmid was selected which contains a small ubiquitin-like  
561 modifier (SUMO) tag as well as a 6x polyhistidine (6X-His) tag, which will facilitate the  
562 solubilization and affinity purification of the recombinant vaccine construct [114]. Also, 6X-  
563 His can facilitate the swift detection of the recombinant vaccine construct in  
564 immunochromatographic assays [115]. The vaccine protein sequence was reverse-translated to  
565 the optimized DNA sequence by the server to which EaeI and StyI restriction sites were  
566 incorporated at the N-terminal and C-terminal sites, respectively. The newly adapted DNA  
567 sequence was then inserted between the EaeI and StyI restriction sites of the pETite vector  
568 using the SnapGene restriction cloning software (<https://www.snapgene.com/free-trial/>) to  
569 confirm the expression of the vaccine [116, 117].

## 570 **2.18. Prediction of the vaccine mRNA secondary structure**

571 Two servers, i.e. Mfold (<http://unafold.rna.albany.edu/?q=mfold>) and RNAfold  
572 (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>), were used for the mRNA  
573 secondary structure prediction. Both of these servers thermodynamically predict the mRNA

574 secondary structures and provide each of the generated structures with minimum free energy  
575 ('G Kcal/mol'). The more stable the folded mRNA is, the lower the minimum free energy and  
576 vice versa [55][118-120]. To analyze the mRNA folding and secondary vaccine structure, the  
577 optimized DNA sequence was first taken from the JCat server and converted via the DNA<-  
578 >RNA->Protein tool (<http://biomodel.uah.es/en/lab/cybertory/analysis/trans.htm>) to a possible  
579 RNA sequence. The RNA sequence was then gathered from the tool and utilized for prediction  
580 into the Mfold and RNAfold servers using the default settings for all the parameters.

581

### 582 **3. Results**

#### 583 **3.1. Protein sequences identification and retrieval**

584 From the NCBI database, the RSV viral strains and the query proteins were identified.  
585 Following that, the four RSV-A and RSV-B Query Proteins including P protein, N protein, F  
586 protein, and mG, were retrieved from the UniProt online database. The UniProt Accession  
587 Number and the length of the query proteins are listed in **Table 1**.

588

589 **Table 01.**

Name of the Virus	Name of the Protein	UniProt Accession Number of the Protein	Length (aa) of the Protein Sequence
RSV-A	P protein	P03421	241
	N protein	P03418	391
	F protein	P03420	574
	mG protein	P03423	298
RSV-B	P protein	O42062	241
	N protein	O42053	391

	F protein	O36634	574
	mG protein	O36633	299

590 List of the proteins with their accession numbers used in the vaccine designing study.

591

592 **3.2. Prediction of antigenicity and analysis of physicochemical**  
593 **properties of the selected proteins**

594 The selected proteins were analyzed for antigenicity and physicochemical properties through  
595 the VaxiJen v2.0 server and ProtParam tool of the ExPASy server, respectively. To be a vaccine  
596 candidate, antigenicity is a prerequisite for a protein or amino acid sequence. All of the selected  
597 proteins were found to be antigenic in VaxiJen v2.0 server at threshold 0.4. In addition, while  
598 P protein and N protein of RSV-A and RSV-B were found to have an acidic theoretical pI (pH  
599 lower than 7), F protein and mG protein were found to have a basic theoretical pI (pH higher  
600 than 7). The protein having an acidic theoretical pI belongs to negatively charged proteins.  
601 Again, in the mammalian cell culture system, all the query proteins had a similar half-life of  
602 30 h and a high aliphatic index (over 60.00) as well. All of the proteins had quite low GRAVY  
603 values (lower than -0.909). P protein of the RSV-A and RSV-B had the highest GRAVY value  
604 of -0.909 and -0.827, respectively. Whereas, the F protein of RSV-A and RSV-B had the lowest  
605 GRAVY value of -0.028 and -0.033, respectively. Furthermore, the F protein of the RSV-A  
606 and RSV-B had the highest aliphatic index of 99.97 and 102.35, respectively. **S1 Table** lists  
607 the results of the analysis of physicochemical properties of all the query proteins.

608 **3.3. Epitope prediction and sorting the most promising epitopes**

609 The RSV-A proteins were selected as models during the prediction of the T-cell and B-cell  
610 epitopes by the IEDB server for the construction of the polyvalent vaccine, meaning that the

611 epitopes were selected using only the RSV-A proteins and then only the fully conserved  
612 epitopes were taken therefore, the epitopes should confer immunity to the selected strains of  
613 both RSV-A and RSV-B. These epitopes were anticipated to induce potential T-cell and B-cell  
614 immune responses after the vaccine administration. Based on the ranking, the top CTL and  
615 HTL epitopes as well as top B-cell epitopes with lengths over ten amino acids were taken into  
616 consideration for further analysis. Following this, a few criteria were selected to filter the best  
617 epitopes which included, high antigenicity, non-allergenicity, non-toxicity, conservancy, and  
618 human proteome non-homology. Furthermore, the cytokine (i.e., IFN- $\gamma$ , IL-4, and IL-10)  
619 inducing ability of HTL epitopes was also considered to determine whether they can produce  
620 at least one of these cytokines. Finally, the epitopes that met these criteria were listed as the  
621 most promising epitopes in **Table 02** and were later used for the construction of the vaccine.  
622 The analysis of transmembrane topology by the TMHMM v2.0 server of the most promising  
623 epitopes revealed that PEFHGEDANNR, SFKEDPTPSDNPFS, EVAPEYRHDSPD,  
624 VFPSDEFDAISQVNEK,  
625 IPNKKPGKTTTKPTKKPTLKTTKKDPKPQTTKSKEVPTTKP were exposed outside.  
626 **S2 Table** listed the potential epitopes of P protein and **S3 Table** listed the potential epitopes of  
627 N protein. The potential epitopes of F protein are listed in **S4 Table** and the potential epitopes  
628 of mG protein are listed in **S5 Table**.

629

630 **Table 02.**

Name of the Protein	MHC class-I epitopes	MHC class-II epitopes	B cell epitopes
P protein	VSLNPTSEK	LGMLHTLVVASAGPT	PEFHGEDANNR
	QTNDNITAR	LHTLVVASAGPTSAR	EVTKESPITSNSTIINPTNETDDTAGNKPQNYQRK
	-	-	SFKEDPTPSDNPFS

	-	-	RNEESEKMAKDTSDDEVSLNPTSEK
N protein	CIAALVITK	EVLTLASLTTEIQIN	EVAPEYRHDSPD
	RSGLTAVIR	-	EYRGTPRNQDLYDA
	SVKNIMLGH	-	-
F protein	KTNVTLSKK	IVIIVILLSLIAVGL	VFPSDEFDASISQVNEK
	KSALLSTNK	VIIVILLSLIAVGLL	-
	IASGVAVSK	EEFYQSTCSAVSKGY	-
	KQLLPIVNK	-	-
	ITIELSNIK	-	-
	LTSKVLDLK	-	-
mG protein	TTTQTQPSK	LSILAMIISTSLIIA	TSQIKNTTPTYLTQNPQLGISPSNPSEITS
	IFIASANHK	TLSILAMIISTSLII	IPNKKPGKKTTKPTKTLKTTKKDPKPQTTSKKEVPTTKP TEEPTINTT
	-	QNPQLGISPSNPSEI	SNTTGNPELTSQ

631 List of the epitopes eventually selected for the construction of the vaccine (selection criteria:  
632 antigenicity, non-allergenicity, non-toxicity, 100 % conservancy and non-homolog to the  
633 human proteome).

634

635 **3.4. Population coverage and cluster analyses of the epitopes and**  
636 **their MHC alleles**

637 The population coverage analysis showed that 85.70% and 87.92% of the world population  
638 were covered by the MHC class-I and class-II alleles and their epitopes, respectively, and  
639 84.62% of the world population was covered by the combined MHC class-I and class-II. While  
640 India had the highest percentage of population coverage for the CTL epitopes (87.56 %) as  
641 well as HTL epitopes (93.51 %), China had the highest percentage of population coverage for  
642 CTL and HTL epitopes in combination (91.80 %) (**Fig 2**).

643

644 **Fig 2.** The result of the population coverage analysis of the most promising epitopes and their  
645 selected MHC alleles

646

647 Cluster analysis of the potential alleles of MHC class I and MHC class II that may interfere  
648 with the predicted epitopes of the RSV query proteins was also conducted. The study was  
649 carried out using the online tool MHCcluster 2.0, which phylogenetically demonstrates the  
650 relation of the allele clusters. **S1 Fig** shows the outcome of the experiment where a strong  
651 interaction is shown in the red zone and a weaker interaction in the yellow zone.

### 652 **3.5. Designing of the multi-epitope subunit vaccine**

653 The most promising T-cell and B-cell epitopes were used to design the multi-epitope vaccine  
654 incorporating adjuvant and appropriate linkers. The hBD-3 was used as an adjuvant to design  
655 the vaccine and the PADRE sequence was also used as a potent inducer of immunity. The  
656 adjuvant was linked with the epitopes by the EAAAK linker. Furthermore, AAY, GPGPG, and  
657 KK were used to associate the epitopes with each other at their appropriate positions as given  
658 in **Fig 3.**

659

660 **Fig 3. (A)** Schematic representation of the potential vaccine construct with linkers (EAAAK,  
661 AAY, GPGPG, and KK), PADRE sequence, adjuvant (hBD-3) and epitopes (CTL, HTL, and LBL)  
662 in a sequential and appropriate manner (B) Sequence of the vaccine protein. The letters in bold  
663 represent the linker sequences.

664

### 665 **3.6. Prediction of antigenicity, allergenicity and Physicochemical** 666 **property analysis of the vaccine**

667 The vaccine protein was observed to be both a potent antigen and a non-allergen. The vaccine  
668 had a high theoretical (basic) pI of 9.75. It had a reasonably adequate half-life in mammalian  
669 cells of 30 h and of more than 10 h in the *E. coli* cell culture system. The GRAVY value of the  
670 vaccine was considered to be significantly negative at -0.362. Additionally, both servers, Sol-  
671 Pro and Protein-sol, have also shown that the vaccine protein is soluble, attesting to its negative  
672 value. The instability index of the protein was found to be less than 40 (27.55), indicating the  
673 vaccine to be quite stable. The extinction coefficient and the aliphatic index of the vaccine were  
674 also found to be high with values, 45770 M<sup>-1</sup> cm<sup>-1</sup> and 80.85, respectively.

### 675 **3.7. Secondary and tertiary structure prediction of the vaccine**

676 The secondary structure of the vaccine protein revealed that the coil structure had the largest  
677 number of amino acids, while the  $\beta$ -strand showed the lowest percentage. The predictions  
678 provided by all four servers are depicted in **Fig 4**. The amino acid percentages of  $\alpha$ -helix,  $\beta$ -  
679 strand, and coil structure of the vaccine protein produced from four different servers are listed  
680 in **Table 03**. All of the servers revealed almost similar predictions and the overall analysis also  
681 showed that the adjuvant generated potential variations in the secondary structure of the  
682 vaccine protein. The vaccine construct's 3D structure was predicted by the RaptorX online  
683 server. The constructed vaccine protein had a surprisingly low p-value of 8.71e-05 in 4  
684 domains, which demonstrated that the accuracy of the proposed 3D structure was significantly  
685 good. Using 1KJ6A as the template from the Protein Data Bank, the homology modeling of  
686 the vaccine construct was completed. Furthermore, the vaccine structure was modeled using  
687 Modeller, as shown in **Fig 5**, to further improve the quality.

#### 688 **Table 03.**

Secondary structure elements	PRISPRED	GOR IV	SOPMA	SIMPA96
$\alpha$ -helix	33.51%	32.98%	30.56%	29.61%

β-strand	16.22%	15.58%	18.46%	14.50%
Coil structure	50.25%	51.44%	50.98%	55.74%

689 Results of the secondary structure analysis of the vaccine construct.

690 **Fig 4.** The results of the secondary structure prediction of the vaccine. (A) PRISPRED  
691 prediction, (B) GOR IV prediction, (C) SOPMA prediction, (D) SIMPA96 prediction.

692 **Fig 5.** (A) The tertiary or 3D structure of the vaccine construct modeled, refined and visualized  
693 by RaptorX, GalaxyWEB server, and BIOVIA Discovery Studio Visualizer v. 17.2  
694 respectively. (B) The results of the Ramachandran plot analysis generated by PROCHECK  
695 server and (C) quality score or z-score graph generated by the ProSA-web server of the refined  
696 vaccine construct. In the Ramachandran plots, the orange and deep yellow colored regions are  
697 the allowed regions, the light yellow regions are the generously allowed regions and the white  
698 regions are the outlier regions and the glycine residues are represented as triangles.

699

### 700 **3.8. Refinement and validation of tertiary structure of the vaccine**

701 The 3D structure of the vaccine protein produced by the RaptorX server was refined to predict  
702 a structure that closely resembles the native protein structure. The refined protein structure was  
703 then validated by evaluating the PROCHECK server-generated Ramachandran plot and the  
704 ProSA-web server-generated z-score. The Ramachandran plot study found that in the most  
705 preferred region, the vaccine protein had 93.5 % of amino acids, while in the additional  
706 approved regions, 5.4 % of amino acids, 0.4 % of amino acids in the generously permitted  
707 regions, and 0.7 % of amino acids in the disallowed regions. In comparison, the z-score of the  
708 engineered vaccine was -6.58, which is beyond the range of all experimentally confirmed X-  
709 ray crystal protein structures from the Protein Data Bank. The protein validation analysis  
710 estimated that there was a reasonably good consistency structure in the distilled form (**Fig 5**).

### 711 3.9. Prediction of conformational B-lymphocytic epitopes

712 The conformational B-cell epitopes of the vaccine protein were predicted using the ElliPro  
713 server which predicts conformational epitopes from tertiary structures of the protein. A score  
714 of 0.50 or higher was selected for the prediction of discontinuous peptides by Ellipro. Three  
715 discontinuous B-cell epitopes were predicted to include 333 amino acid residues, with values  
716 ranging from 0.506 to 0.675. The size of the conformation epitopes varied from 4 to 394  
717 residues. Three-dimensional representation of conformational B cell epitopes of the designed  
718 multi-epitope-based RSV vaccine and the epitope residues are shown in **Fig 6** and listed in **S6**

719 **Table.**

720

721 **Fig 6.** Graphical representations of the predicted conformational B-cell epitopes of the modeled  
722 vaccine indicated by yellow coloured ball-shaped structures.

723

### 724 3.10. Vaccine protein disulfide engineering analysis

725 The disulfide bonds of the vaccine structure were predicted using the DbD2 server in protein  
726 disulfide engineering. Based on certain classification criteria, the server recognizes pairs of  
727 amino acids with the ability to form disulfide bonds. Only those amino acid pairs that had bond  
728 energy smaller than 2.2 kcal/mol were chosen in this experiment. Three pairs of amino acids  
729 with bond energy below 2.2 kcal/mol were provided by the RSV: 23 Cys and 38 Cys, 414 Ala  
730 and 414 Lys, and 513 Tyr- 516 Thr. The selected pairs of amino acids have formed the mutant  
731 vaccine in the DbD2 server, which contains potential disulfide bonds within (**S2 Fig**). This  
732 indicates the probable stability of the designed multi-epitope vaccine construct.

### 733 **3.11. Post-translational modification analysis**

734 The posttranslational modification analysis was performed to see whether the vaccine construct  
735 would undergo any substantial changes after being administered to mammalian cells. Four N-  
736 glycosylation sites and sixty-three O-glycosylation sites were predicted in the vaccine construct  
737 sequence. The findings suggest that a significant amount of glycosylation may have occurred  
738 inside the predicted vaccine construct, which might improve the vaccine's efficacy and  
739 immunogenicity. In addition, the vaccine protein sequence has ninety-six phosphorylated  
740 residues (i.e., serine residues (S), threonine (T), and tyrosine (Y) phosphorylation sites)  
741 according to the NetPhos v2.0 server output. The server-provided plots containing the N-  
742 glycosylation sites and phosphorylation are given in **S3 Fig.**

### 743 **3.12. Analysis of protein-protein docking**

744 Protein-protein docking analysis was performed to demonstrate the vaccine's ability to interact  
745 with various crucial molecular immune components i.e., TLRs. When docked using ClusPro  
746 2.0, it demonstrated very high binding affinities with all its targets (TLRs). It has been further  
747 studied using the ZDOCK server where the vaccine protein also displayed very strong  
748 interaction with the TLRs. The lowest energy level obtained for docking between the vaccine  
749 construct and TLR-1, TLR-2, TLR-3, TLR-4, and TLR-9 were -986.1, -1236.7, -1084.4, -  
750 1260.8, and -1226.4, respectively. The lowest energy level between the vaccine and TLRs  
751 indicated the highest binding affinity.

### 752 **3.13. Molecular dynamics simulation studies and MM-PBSA**

#### 753 **calculations**

754 MD simulation is an effective method for the analysis of biological systems and it provides  
755 many mechanistic insights into the possible behavior of the system under a simulated biological

756 environment [121]. Gromacs 2020.4 was used to carry out the production phase MD and the  
757 analysis of resulting trajectories was undertaken to understand the structural properties, and  
758 interaction between different TLRs and the predicted vaccine protein at a molecular level. The  
759 snapshots of equilibrated structures of each TLR-vaccine complex and the snapshots of the last  
760 trajectories are shown in **Fig 7**. The visual inspection of trajectories at different time intervals  
761 suggested that the side chains of the vaccine make different interactions with chain A of all  
762 TLRs except TLR1, where vaccine side chains were found to be interacting with side chains  
763 of both A and B chains.

764

765 **Fig 7.** Snapshots of equilibrated (initial) systems and last trajectories. Vaccine bound  
766 complexes of A) TLR1, B) TLR2, C) TLR3, D) TLR4, and E) TLR9 (For each snapshot the  
767 surface representation and cartoon representations are shown)

768

769 Root mean square deviations (RMSD) analysis gives insights into how the backbone atoms  
770 move relative to the initial equilibrated positions. Lower the RMSD, better the stability of the  
771 corresponding system. In the present work, we measured the RMSD in backbone atoms of  
772 entire protein-protein complexes of TLRs with a vaccine. **Fig 8A** shows the RMSD in the  
773 investigated systems. The evaluation of root means square fluctuations (RMSF) provides  
774 insights into the possible changes in the secondary structure of protein under investigation. In  
775 the present work, the RMSF in the side chain atoms of residues in each system was measured.  
776 As the TLR-vaccine systems have multiple chains, the RMSF evaluation is performed on each  
777 chain of the complex to understand which residues are involved in the key contacts. The RMSF  
778 in the TLR side-chain atoms is shown in **Fig 8B**. The RMSF in other chains in each of the  
779 TLRs is given in **S4 Fig**. The analysis of radius of gyration (Rg) provides the overall  
780 measurement of compactness of the system [122]. The results of total Rg are shown in **Fig 8C**.

781 Analysis of non-bonded interactions such as hydrogen bonds is quite challenging in protein-  
782 protein complexes. The side chains of the proteins participate in hydrogen bond interactions.  
783 The hydrogen bond analysis of all the trajectories was performed with the h-bond module of  
784 Gromacs, while the key residues at the interface of the TLR chain and vaccine chain were  
785 analyzed through the chimeraX program [123]. The results of the hydrogen bond analysis are  
786 shown in **S5 Fig**.

787

788 **Fig 8:** Results of the (A) Root mean square deviations in the investigated systems, (B) Root  
789 mean square fluctuations in the side chain atoms of vaccine, and (C) Radius of gyration of the  
790 vaccine.

791

### 792 **3.14. Immune simulation studies**

793 The immune simulation study of the designed vaccine was conducted using the C-ImmSimm  
794 server which forecasts the activation of adaptive immunity as well as the immune interactions  
795 of the epitopes with their specific targets [63]. The analysis exhibited that the primary immune  
796 reaction to the vaccine could be stimulated substantially after administration of the vaccine, as  
797 demonstrated by a steady rise in the levels of different immunoglobulins i.e., (IgG1 + IgG2,  
798 and IgG + IgM antibodies) (**Fig 9A**). It was also expected that the concentrations of active B  
799 cells (**Fig 9B** and **Fig 9C**), plasma B cells (**Fig 9D**), helper T cells (**Fig 9E** and **Fig 9F**), and  
800 cytotoxic T cells (**Fig 9H** and **Fig 9I**) could steadily increase, reflecting the vaccine's capacity  
801 to create a very high secondary immune response and healthy immune memory. However, **Fig**  
802 **9G** demonstrates that the concentration of regulatory T cells would gradually decrease  
803 throughout the phases of the injections, which represents the decrease in suppression of  
804 vaccine-induced immunity by regulatory T cells [124].

805 In comparison, the rise in macrophage and dendritic cell concentrations showed that these  
806 APCs had a competent presentation of antigen (**Fig 9J** and **Fig 9K**). The simulation result also

807 predicted that the constructed vaccine could generate numerous forms of cytokines, including  
808 IFN- $\gamma$ , IL-23, IL-10, and IFN- $\beta$ ; some of the most critical cytokines for producing an immune  
809 response to viral infections (**Fig 9L**). Therefore, the overall immune simulation analysis  
810 showed that after administration, the proposed polyvalent multi-epitope vaccine would be able  
811 to elicit a robust immunogenic response.

812 **3.15. Codon adaptation, *in silico* cloning, and interpretation of the**  
813 **vaccine mRNA secondary structure**

814 The protein sequence of the vaccine was adapted by the JCcat server for *in-silico* cloning and  
815 plasmid construction. The CAI value was found to be 0.98, suggesting that the DNA sequences  
816 contained a larger proportion of the codons most likely to be included in the target organism's  
817 (K12 strain of *E.coli*) cellular machinery [118, 119]. Furthermore, GC content of the formed  
818 sequence was found to be 50.23 %, within the desired range. The graph demonstrating the  
819 sequence after codon adaptation is shown in **S6 Fig**. Following codon adaptation, the projected  
820 vaccine DNA sequence was inserted between the EaeI and StyI restriction sites into the pETite  
821 vector plasmid. The plasmid includes SUMO and 6X H tag sequences that are required to  
822 promote the vaccine's purification during downstream processing [125]. The newly built  
823 recombinant plasmid has been designated as "Cloned\_ pETite" (**Fig 10**). Thereafter, the Mfold  
824 and RNAfold servers predicted the secondary structure of the vaccine mRNA. A minimum free  
825 energy score of -549.30 kcal/mol was produced by the Mfold server, which was consistent with  
826 the prediction of the RNAfold server that also predicted a minimum free energy of -526.30  
827 kcal/mol. In **S7 Fig**, the vaccine mRNA secondary structure is depicted.

828

829 **Fig 10.** *In-silico* cloning of the vaccine sequence in the pETite plasmid vector. The codon  
830 sequence of the final vaccine is presented in red generated by the JCat server. The pETite  
831 expression vector is in black.

832

833 **4. Discussion**

834 hRSV is conventionally the most prevalent cause of human LRTIs, known to infect individuals  
835 from all age groups but more commonly newborns and children. Implicated infections of RSV  
836 contribute to affecting and killing numerous people all over the globe, yet no pre-existing  
837 authorized vaccine is recognized as an effective measure to prevent RSV infections. Vaccines  
838 are extensively used to control and prevent diseases caused by a variety of pathogens across  
839 the world. Conventional methods are primarily used for vaccine development and manufacture,  
840 despite the associated disadvantages of being expensive and time-consuming [126]. In contrast  
841 to traditional vaccine production strategies, today's cutting-edge research and technology as  
842 well as the availability of knowledge about the genome and proteome of almost all viruses and  
843 organisms, facilitate the design and development of novel peptide-based subunit vaccines.  
844 Subunit vaccines have the benefit of being able to eliminate toxic and immunogenic  
845 components of an antigen during a vaccine design study, making the vaccine safe to administer  
846 in people. Subunit vaccines include a limited amount of viral particles that cause patients to  
847 develop protective immunity. A subunit vaccine is a cost-efficient and effective way to prevent  
848 health concerns [127-129]. As a result, bioinformatics and immunoinformatics techniques have  
849 been developed and widely utilized to design novel subunit vaccines that are safe, effective,  
850 efficient, and low-cost alternatives to current preventive measures [130, 131].

851

852 The narrated experiment utilized immunoinformatics methods to design a blueprint of a  
853 polyvalent epitope-based vaccine against the antigenic subgroups, RSV-A and RSV-B,  
854 targeting four distinctive proteins which include - P protein, N protein, F protein, and mG  
855 protein. Antigenicity and physicochemical properties of the proteins were predicted, where all  
856 the proteins identified were shown to be antigenic; a requirement for the use of a target protein  
857 in epitope-based vaccine construction. The theoretical pI represents the pH at which there is no  
858 net effective charge and mobility in a protein as well as predicts whether a protein is basic or  
859 acidic [132]. The instability index of a protein represents the likelihood of that specific  
860 compound being stable, and a compound with an instability index greater than 40 is deemed  
861 unstable [133]. The aliphatic index measures the relative amount of amino acids occupied by  
862 aliphatic amino acids in its side chains [134]. The high aliphatic index also indicates the  
863 improved thermal stability of a protein [135]. All of the query proteins had a high extinction  
864 coefficient and theoretical half-life of 30 h in mammalian cells. The extinction coefficient  
865 indicates the ability of a protein to absorb light at a certain wavelength and a higher extinction  
866 coefficient represents the higher absorbance of light by the protein [136, 137]. The GRAVY  
867 value represents a compound's hydrophilic or hydrophobic traits. The GRAVY negative value  
868 reflects hydrophilic characteristics, while the GRAVY positive value reflects the hydrophobic  
869 characteristics of the compound [138, 139]. Since all query proteins were found to be quite  
870 antigenic having a high aliphatic index and extinction coefficient, as well as negative GRAVY  
871 values, they were expected to be thermostable, high light-absorbing as well as hydrophilic in  
872 nature. Overall, the physiological property analyses of the proteins revealed satisfactory results,  
873 desired for the epitope predictions. CTL, HTL, and LBL epitopes are some mandatory  
874 constituents for a multi-epitope subunit vaccine, known to stimulate or activate the cytotoxic  
875 T-cells, helper T-cells, and B-cells to generate an effective host immune response [140].  
876 Cytotoxic T-cells can recognize the foreign antigens while helper T-cells recruit the other

877 immune cells including B-cells, macrophages, and even cytotoxic T-cells to ensure the  
878 generation of immune responses [38, 48]. In addition, B-cells mediate the humoral immune  
879 response by producing immunoglobulins or antibodies that are antigen-specific [50][141, 142].

880 Once the vaccine protein reaches the host antigen-presenting cells (APC), they are processed,  
881 and the T cell epitopes are proteolytically cleaved off the protein, which is then represented by  
882 MHC molecules on the surface of APCs, exposing them to T cell receptors [143]. MHC class  
883 I molecules represent endogenous antigens often referred to as epitopes, such as intracellular  
884 proteins of a pathogen (e.g., bacteria or virus) or any tumor-inducing proteins whereas, MHC  
885 class II molecules represent exogenous epitopes. Furthermore, the antigen region that binds to  
886 the immunoglobulin or antibody is referred to as the B-cell epitope. These B-cell epitopes can  
887 be found in any exposed solvent area of the antigen and can be of various chemical types. The  
888 majority of antigens, however, are proteins, which are the targets of epitope prediction  
889 algorithms. The goal of B-cell epitope prediction is to ensure a more convenient method to  
890 identify B-cell epitopes, to substitute antigen for antibody production by the plasma B-cells, or  
891 conduct structure-function studies. Thus, antibodies can recognize any area of the antigen that  
892 has been exposed to solvents. B-cell epitopes can be split into two categories: linear and  
893 conformational; conformational B-cell epitopes are made up of patches of solvent-exposed  
894 atoms from residues that are not always sequential, while LBL epitopes are made up of  
895 sequential residues. Antibodies that identify LBL epitopes can recognize denatured antigens,  
896 but denaturing the antigen causes conformational B-cell epitopes to lose their recognition  
897 [144]. T-cell and B-cell epitopes have been predicted for the selected RSV proteins using the  
898 IEDB server. The most conserved epitopes with high antigenicity, non-allergenicity, and non-  
899 toxicity were screened for designing the vaccine construct. The broad-spectrum activity of the  
900 vaccine over the selected strains of both RSV-A and RSV-B viruses was assured by the  
901 conservancy of the epitopes.

902 Furthermore, the cytokine-producing ability was considered as a criterion to screen the HTL  
903 epitopes desired for designing the vaccine. Inflammatory mediators, such as cytokines and  
904 chemokines have been linked to RSV pathogenesis. They may be divided into two groups  
905 depending on how they affect immune cells: pro-inflammatory and anti-inflammatory  
906 chemicals [144]. Interleukin (IL)-1, tumor necrosis factor-alpha (TNF- $\alpha$ ), interferon-gamma  
907 (IFN-  $\gamma$ ), and interleukin-6 (IL-6) are pro-inflammatory cytokines [145-147]. IL-10 and IL-12  
908 are anti-inflammatory cytokines [148, 149]. IFN- $\gamma$  , on the other hand, has a dual role during  
909 RSV infection; it is essential to decrease viral multiplication while simultaneously inhibiting  
910 airway blockage [150]. IFN- $\gamma$  is well-known for its fundamentally safe responses and potential  
911 to stop viral multiplication [151, 152]. Additionally, IL-4 plays important role in regulating the  
912 responses of lymphocytes, myeloid cells, and non-hematopoietic cells. In T-cells, IL-4 induces  
913 the differentiation of naïve CD4 T cells into Th2 cells, and in B cells, IL-4 drives the  
914 immunoglobulin (Ig) class switch to IgG1 and IgE, and in macrophages, IL-4, as well as IL-  
915 13, induce alternative macrophage activations [153]. Consequently, cytokines including IFN-  
916  $\gamma$ , IL-10, and IL-4 were considered essential during the prediction of the HTL epitopes of the  
917 vaccine which, after administration, might play a crucial role in creating a network between  
918 immune system cells [63]. The population coverage analysis was performed in the following  
919 step. As HLA allelic distribution varies between geographical regions and ethnic groups  
920 throughout the world, it is paramount to consider population coverage while designing a viable  
921 epitope-based vaccine that is pertinent to global populations. According to the population  
922 coverage analysis, the MHC Class-I and Class-II alleles and their epitopes covered 85.70 %  
923 and 87.92 % of the global population, respectively, while the combined MHC Class-I and class-  
924 II alleles and their epitopes covered 84% of the world population. When compared to the  
925 overall population, selected epitopes exhibited a greater individual percentage cover which  
926 indicates the potential worldwide effects of the vaccine against RSV infections.

927

928 The most promising epitopes had been conjugated by specific linkers, i.e. EAAAK, AAY, KK,  
929 and GPGPG. An innate antimicrobial peptide, hBD-3 was used as an adjuvant during the  
930 construction of the vaccine. An adjuvant is considered crucial for designing a subunit vaccine  
931 because it improves the traits of antigenicity, immunogenicity, durability, and longevity of the  
932 subunit vaccine [154]. The hBD-3 was chosen as it could potentially induce TLR-dependent  
933 expression of the co-stimulatory molecules - CD80, CD86, and CD40 on the surface of  
934 monocytes and myeloid dendritic cells [155]. Moreover, by forming a protective barrier of  
935 immobilized surface proteins, hBD-3 can prevent the fusion of the virus [156]. Furthermore, it  
936 activates the APCs through TLR1 and TLR2 [157], stimulates IL-22 [158], TGF- $\alpha$  [159, 160],  
937 and IFN- $\gamma$  [161, 162]. It also facilitates the chemotaxis of immature DCs and T cells through  
938 its interaction with chemokine receptor 6 (CCR6), as well as the chemotaxis of monocytes  
939 through its interaction with CCR2 [163]. This peptide also promotes and activates myeloid  
940 DCs and natural killer (NK) cells [157, 162].

941 Alongside the adjuvant, to strengthen the immunogenic reaction of the vaccine, the PADRE  
942 sequence was also incorporated. The antigenicity, allergenicity, and physicochemical  
943 properties of the constructed vaccine were subsequently identified, which revealed the vaccine  
944 protein to be desirable for further modeling refinement and validation processes. The vaccine  
945 protein was predicted with a theoretical pI of 9.75, indicating that the vaccine protein is basic  
946 and it might belong to the positively charged proteins [132]. The vaccine's GRAVY value was  
947 predicted to be quite negative (-0.362), indicating that the vaccine protein is hydrophilic [138,  
948 139]. Moreover, the instability index was determined to be less than 40 (27.55), implying that  
949 the vaccine is fairly stable. The extinction coefficient which is representative of the light-  
950 absorbing nature, and the aliphatic index which denotes the high stability of the vaccine protein,  
951 were both found to have high values at 45770 M<sup>-1</sup> cm<sup>-1</sup> and 80.85, respectively [133]. Using

952 different online tools to predict the secondary and tertiary structure of the vaccine, it was  
953 revealed that the adjuvant sequences had produced some substantial changes in the predicted  
954 vaccine construct. Furthermore, the secondary structure analysis showed that the vaccine  
955 protein sequence was abundant with the coiled regions as well as the very low amount of  $\beta$ -  
956 strand. This indicates the higher stability and conservation of the predicted vaccine model. The  
957 tertiary structure was modeled and refined once the secondary structure was determined. The  
958 tertiary structure prediction of the vaccine protein revealed a p-value of 8.71e-05 in four  
959 domains of the protein, indicating that the predicted 3D structure was quite accurate. The  
960 quality of the vaccine was greatly enhanced following refinement in the context of GDT-HA,  
961 MolProbity, Rama favored amino acid percentage, and z scores, according to the tertiary  
962 structure refinement and validation study. With only a few amino acids in the outlier regions,  
963 the refined structure showed a very high Rama favored amino acid percentage. Following that,  
964 the refined structure was used for disulfide engineering. Furthermore, disulfide engineering of  
965 the vaccine construct has been conducted to increase its stability using the DbD2 v12.2 servers.  
966 The server can determine the B-factor of areas involved in disulfide bonding as well as identify  
967 potential disulfides that increase the protein's thermal stability [92]. All residue pairings in a  
968 given protein structural model are quickly analyzed for closeness and geometry compatible  
969 with disulfide formation, assuming the residues have been changed to cysteines. Similarly, the  
970 experimental result shows residue pairings that match the specified requirements. Engineered  
971 disulfides have been shown to improve protein stability and aid in the study of protein dynamics  
972 and interactions [94]. Three pairs of amino acids have been found by the server which is  
973 predicted to improve the stability of the vaccine construct thereby. However, the vaccine's  
974 possible effectiveness and immunological responses may be reduced if posttranslational  
975 modification, such as glycosylation and phosphorylation, is overlooked during vaccine  
976 development. Glycosylation is a chemical modification of macromolecules in which

977 carbohydrate moieties are covalently bonded to the N or C terminals of lipids and proteins  
978 molecules, resulting in N-linked and O-linked glycosylation [164]. Previous studies have found  
979 that glycosylation significantly improves vaccine immunogenicity when compared to non-  
980 glycosylated vaccinations, and clinical trials are now ongoing [165]. Furthermore,  
981 phosphorylation is the process of adding a phosphate group to macromolecules. Eukaryotes  
982 have a greater frequency of occurrence of posttranslational modifications. Serine and threonine  
983 are two important phosphorylation sites. Phosphorylated peptides or epitopes (synthetic or  
984 natural) are known to be better recognized by cytotoxic T cells, i.e. MHC Class I molecules,  
985 and are therefore directly implicated in the production of particular immune responses [166].  
986 As a result, it is hypothesized that the vaccine construct predicted with multiple  
987 posttranslational modifications will create an efficient immunogenic response against the virus  
988 following vaccine administration.

989 Furthermore, one of the predominant necessities and approaches to designing an effective  
990 vaccine is the molecular docking process. It concludes the probability of contact between the  
991 vaccine and other networking proteins, i.e. TLRs may occur during initial immune response.  
992 TLRs, which are found on leukocytes and in tissues, play a major role in innate immunity  
993 activation by identifying invading pathogens, including viruses like RSV, and sending out  
994 signals that promote inflammation-related components [167]. TLRs, such as TLR2, TLR1,  
995 TLR6, TLR3, and TLR4, are found on leukocytes and can interact with RSV to boost immune  
996 responses [168]. Within the lungs, TLR2 interactions with RSV increase neutrophil migration  
997 and dendritic cell activation. TLR2 exists as a heterodimer complex with either TLR1 or TLR6  
998 on the surface of immune cells and tissues [169]. According to genetic analysis and vaccine  
999 studies, TLR2 signaling appears to be critical in RSV recognition [170-172]. TLR2 and TLR1  
1000 or TLR2 and TLR6 complexes can recognize RSV, and greatly enhance early innate  
1001 inflammatory responses [173-175]. Previous research has also suggested that the signals

1002 generated by TLR2 and TLR6 activation are critical for viral replication control [168]. TLR3  
1003 and TLR4 signaling support the T helper type 1 (Th1) responses, whereas T helper type 2 (Th2)  
1004 responses are favored by TLR2/1, and TLR2/6 signaling [177]. Th1 cells help with responses  
1005 to intracellular pathogens, whereas Th2 cells handle parasitic infections and allergies [178,  
1006 179]. The F glycoprotein of RSV has been reported to induce primarily a Th1-type immune  
1007 response through the interaction with TLR4 [180, 181]. On the other hand, TLR9 has been  
1008 found to improve vaccine immunogenicity and decrease vaccine-enhanced illness during FI-  
1009 RSV immunization. Furthermore, immunomodulation generated by TLR9 agonists verifies  
1010 TLR agonists' adjuvant potential after RSV immunization [182]. Finally, the targeted TLRs are  
1011 all involved in pattern recognition and the innate immune response to RSV, which results in  
1012 the production of proinflammatory cytokines and chemokines [183]. TLRs which are critical  
1013 in the RSV pathogenesis were considered for the docking analysis. Thus, TLR-1, TLR-2, TLR-  
1014 3, TLR-4, and TLR9 have been docked with the vaccine protein which revealed each of the  
1015 TLRs to obtain very low binding energy in different servers and to show strong interaction with  
1016 the vaccine protein according to the results given in the docking analysis. It is evident from the  
1017 docking analysis that the designed vaccine would have a strong affinity with all the target  
1018 TLRs, leading to the possibility that a strong immune response might be induced by the vaccine  
1019 after administration.

1020 In response to external forces exerted by its surrounding environment, MD simulation  
1021 examines the motion and changes in the state of a target protein molecule or complex. In this  
1022 experiment, to get a better insight into their molecular stability, the five docked vaccine-TLR  
1023 complexes were simulated. The experiment revealed that the structures retained appropriate  
1024 levels of deviation. The TLR1-vaccine complex was found to have the least RMSD compared  
1025 to other complexes; the TLR4-vaccine complex had higher RMSD despite having a similar  
1026 number of chains. The result of RMSD represents the stability of TLR1 and TLR9 vaccine

1027 complexes. Despite 3 chains *viz.* Chain A, chain B, and vaccine chain in the TLR1-vaccine  
1028 complex, the resultant RMSD, suggest reasonable stability. Furthermore, The RMSF analysis  
1029 suggests the residues in the range 190-240 and 480-530 are having large magnitudes of  
1030 fluctuations in all the complexes. The TLR3-vaccine complex showed slightly larger  
1031 fluctuating side chains than other complexes. The results of the total Rg analysis suggest that  
1032 the total Rg of the TLR1-vaccine complex almost remained constant throughout the simulation.  
1033 The total Rg of the TLR4-vaccine complex was found lower; however, it deviated throughout  
1034 the simulation. Though both these TLRs have two chains of proteins along with vaccine chains,  
1035 TLR1 Rg seemed quite stable, while the other TLR systems (TLR2, TLR3) also seemed to be  
1036 quite compact, and there were no evident secondary structural changes in these TLRs. In the  
1037 non-bonded interaction analysis, in the case of TLR1-vaccine complex, it is found that around  
1038 10 hydrogen bonds were formed till around 50 ns MD time interval, which steadily lowers to  
1039 around 5 hydrogen bonds till 80 ns and thereafter rises to 10 hydrogen bonds. The TLR2-  
1040 vaccine complex has around 7 hydrogen bonds being constantly formed throughout the MD  
1041 simulation, while TLR3 and TLR9 complexes with vaccines have around 10 hydrogen bonds  
1042 constantly formed. These complexes have strong hydrogen bond networks at the interface of  
1043 TLRs and vaccines. The TLR4-vaccine complex has around 5 hydrogen bonds formed which  
1044 are fewer in number compared to other systems. Actual residues involved in the hydrogen bond  
1045 formation as investigated in the last trajectory are tabulated in the **S7 Table**.

1046  
1047 The immune simulation analysis of the proposed vaccine demonstrated that the vaccine could  
1048 induce an immune response compatible with the natural host immune system. Both humoral  
1049 and cell-mediated responses may be activated by the vaccine, as shown by an elevation in the  
1050 levels of memory B cells, plasma B cells, cytotoxic T cells, helper T cells, and various  
1051 antibodies. Adaptive immunity is an immunity that occurs after exposure to an antigen either

1052 from a pathogen or a vaccination. A vaccine can generate adaptive immunity against the  
1053 pathogen by which it may restrict or prevent the infection. The vaccine-provided activation of  
1054 helper T cells resulted in strong adaptive immunity [184-186]. Again, a very strong antigen  
1055 presentation was also demonstrated in the simulation study by the rise in the concentration of  
1056 APCs such as macrophages and dendritic cells. In addition, enrichment in the cytokine profile  
1057 that plays a crucial role in providing broad-spectrum immunity against viral invasions [186-  
1058 189] has also been identified in the analysis [66]. Moreover, the gradual increase in the level  
1059 of different mucosal immunoglobulins i.e. IgG1 + IgG2, and IgG + IgM antibodies throughout  
1060 the vaccine doses or injections were also predicted. The mucosal immune system is the  
1061 dominant part of the immune system, having developed to protect the mucosae in the upper  
1062 respiratory tract, which are the primary sites of respiratory infection. Being a respiratory virus,  
1063 RSV initially affects the upper respiratory tract, due to which the immune system may be  
1064 stimulated against the virus predominantly at the mucosal surfaces [190]. Previous clinical  
1065 studies also reported IgG and IgM to be significant role players against RSV infections [191].  
1066 Furthermore, the simulation analysis revealed that the concentration of regulatory T cells would  
1067 gradually decrease throughout the phases of the vaccine doses which indicates the potential  
1068 decrease in suppression of vaccine-induced immunity by regulatory T cells [192].  
1069 Hence, the proposed vaccine construct is predicted to produce an effective immunogenic  
1070 response after the vaccine injections, according to the analysis of the immune simulation. The  
1071 codon adaptation and subsequent *in silico* cloning studies were conducted to identify the  
1072 potential codons required for the generation of a recombinant plasmid that could be used to  
1073 express the vaccine in the *E. coli* strain K12, leading to the mass manufacturing efforts of the  
1074 vaccine in the near future. The *E. coli* cell culture system is considered to be the majorly  
1075 recommended system for the production of recombinant proteins at a mass level. In the codon  
1076 adaptation analysis, the obtained results were significantly good with a CAI value of 0.98 and

1077 a GC content of 50.23 %, since any CAI value above 0.80 and a GC content of 30% to 70%  
1078 are considered to be the most promising scores [34, 186, 193]. Following this, the optimized  
1079 vaccine DNA sequence was inserted into the pETite plasmid vector using Snapgene restriction  
1080 cloning software. The pETite plasmid vector contains SUMO tag and 6X His tag which might  
1081 be fused with the vaccine codon sequence during the *in silico* cloning process. This may lead  
1082 to the expression of these tags within the protein itself, which could promote the purification  
1083 and downstream processing of the vaccine. Prediction of the stability of the vaccine mRNA  
1084 secondary structure using the Mfold and RNAfold servers provided with the negative and much  
1085 lower minimal free energies of -549.30 and -526.30 kcal/mol, respectively. The lower minimal  
1086 free energy is often considered better than the higher maximal free energy score which indicates  
1087 the protein to be more stable. It can, therefore, be reported that the predicted vaccine could be  
1088 very stable upon transcription [133]. Overall, this study suggests that the proposed vaccine  
1089 peptide could be utilized as a potential and successful protective measure against both RSV-A  
1090 and RSV-B subtypes. However, to eventually validate its immunogenicity, efficacy, stability,  
1091 safety, and various biophysical characteristics, further research approaches, and  
1092 implementations are recommended.

1093

1094 **5. Conclusion**

1095 RSV is predominantly one of the major contributors of diseases of the LRTI, including  
1096 pneumonia and bronchiolitis, responsible for infecting people of all ages as well as  
1097 immunocompromised individuals with a high infection rate. Millions of individuals eventually  
1098 end up being diagnosed with this virus every year, and a huge proportion of them require to be  
1099 hospitalized. While research for an effective countermeasure to tackle this virus has been  
1100 ongoing for the past few decades, no approved vaccine is still commercially available.

1101 Moreover, the antiviral drugs now available often struggle to show any effective outcomes  
1102 during therapy. Therefore, a potential epitope-based polyvalent vaccine against both forms of  
1103 RSV, RSV-A, and RSV-B, was designed in this research using the techniques of  
1104 immunoinformatics and *in-silico* biology. The vaccine included the T-cell and B-cell epitopes  
1105 that were 100% conserved; they could therefore be efficient against the two selected viruses.  
1106 In addition, high antigenicity, non-allergenicity, and non-toxicity as well as non-homology (to  
1107 the human proteome) were also considered to be the criteria for choosing the most promising  
1108 epitopes for the final construction of the vaccine, so that the vaccine could deliver a very strong  
1109 immunogenic response without triggering any adverse reaction inside the body. The results of  
1110 the various analyses conducted in the study revealed that the polyvalent vaccine should be very  
1111 safe, efficient, and responsive to use. The tools utilized in this study are well accepted and yield  
1112 highly accurate results. Therefore, the findings of this study can point researchers in the  
1113 direction of novel vaccine development tactics. Researchers could investigate the predicted  
1114 epitopes and their probable immunogenic response elicited in the host system when looking  
1115 into further subunit vaccine development or other prevention strategies against RSV infection.  
1116 However, as all these predictions were focused solely on computational techniques, it is  
1117 important to perform further wet laboratory-based experiments to validate the findings of this  
1118 analysis. With high-cost criteria and numerous drawbacks in improving the preparation of a  
1119 live, attenuated, or inactivated vaccine for such highly infectious agents, candidates for peptide-  
1120 based vaccines, such as the one designed in this study, maybe comparatively cheap and an  
1121 efficient alternative to reach the entire world as a polyvalent vaccine to fight the challenge of  
1122 RSV infections.

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1129 **7. Declarations**

1130 **Ethics approval and consent to participate**

1131 Not Applicable

1132 **Consent for publication**

1133 Not Applicable

1134 **Availability of data and material**

1135 All the data generated during the experiment are provided in the manuscript/supplementary  
1136 material.

1137 **Competing interests**

1138 The authors declare that they have no conflict of interest regarding the publication of the paper.

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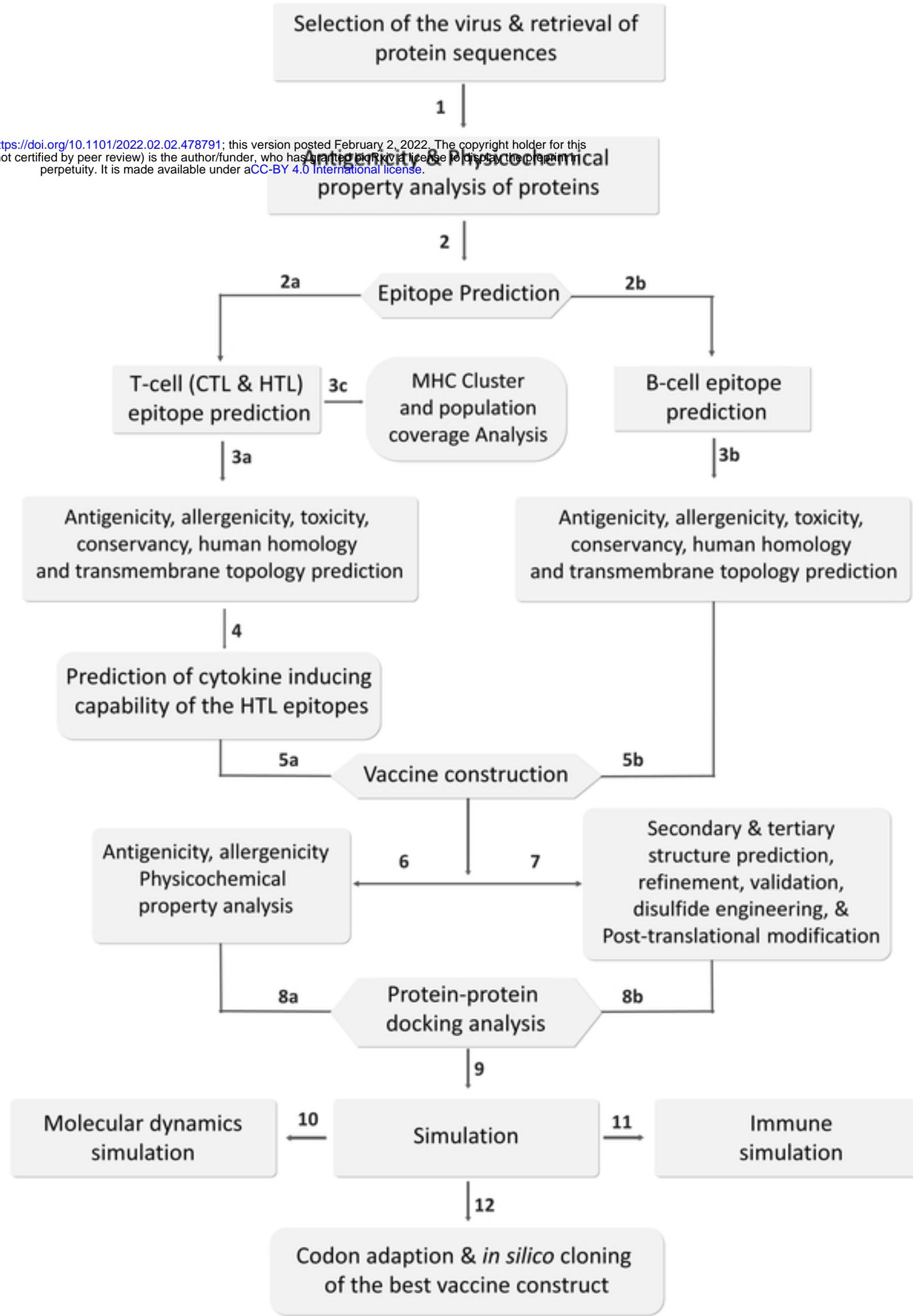
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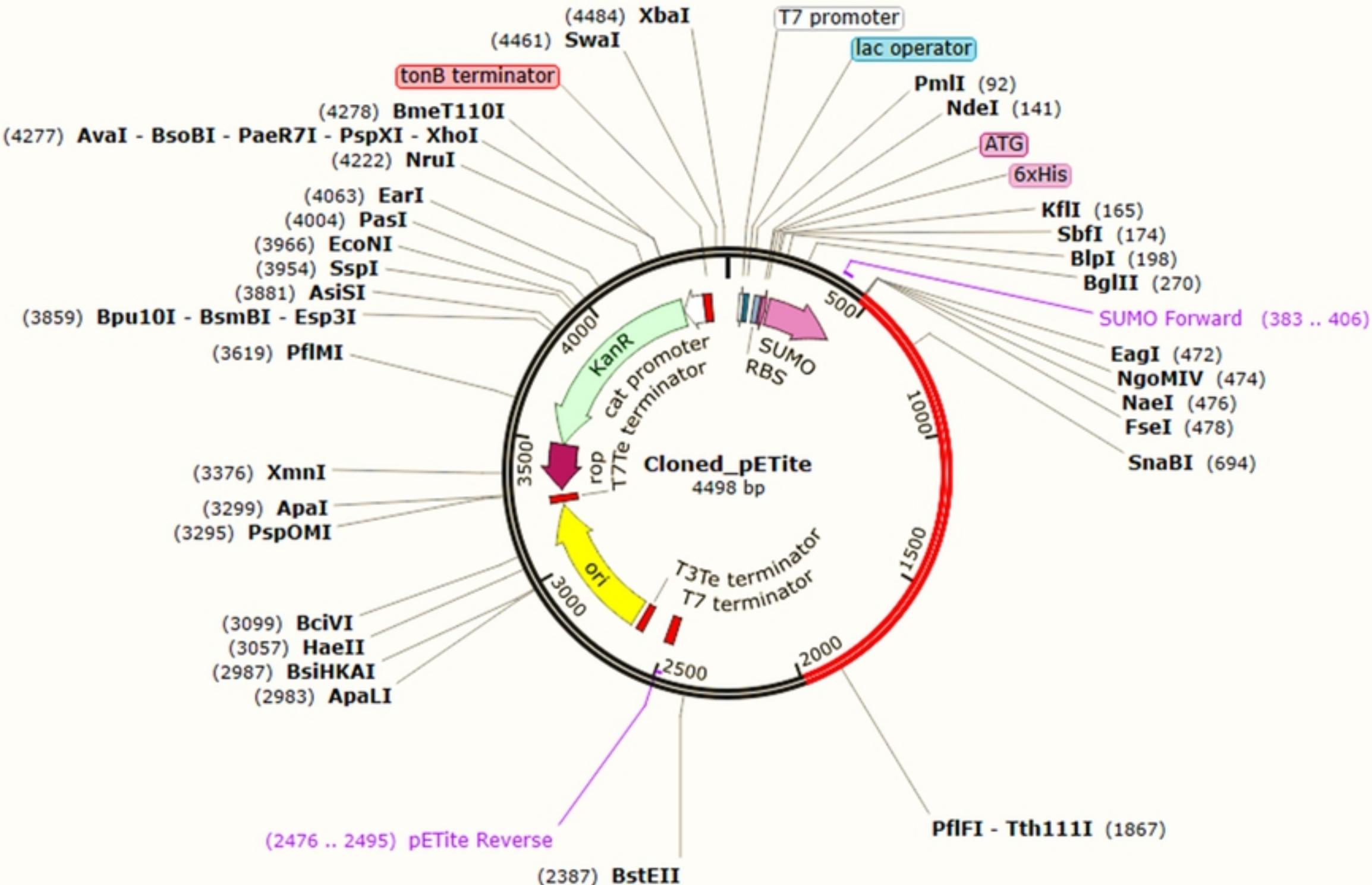


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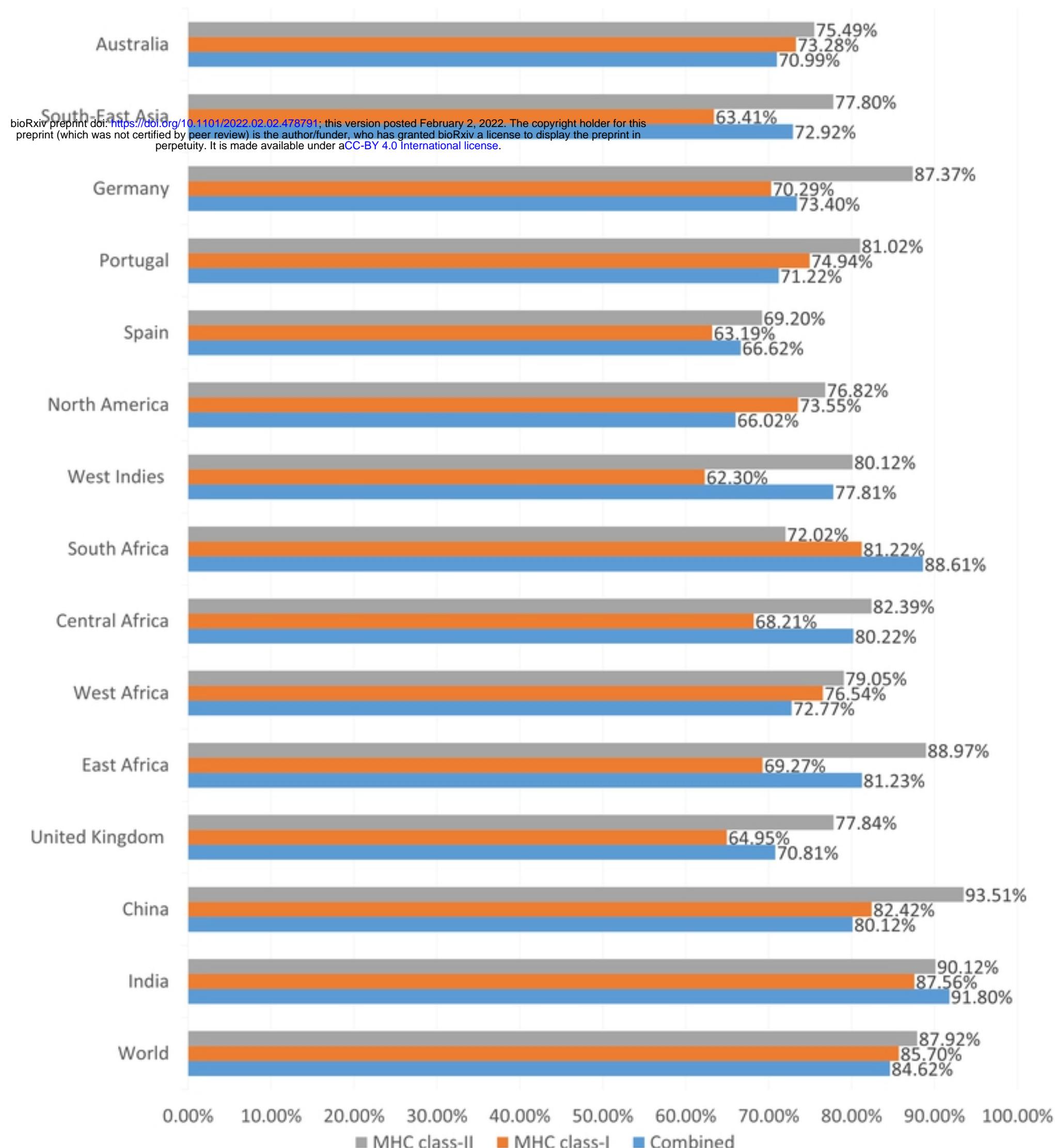
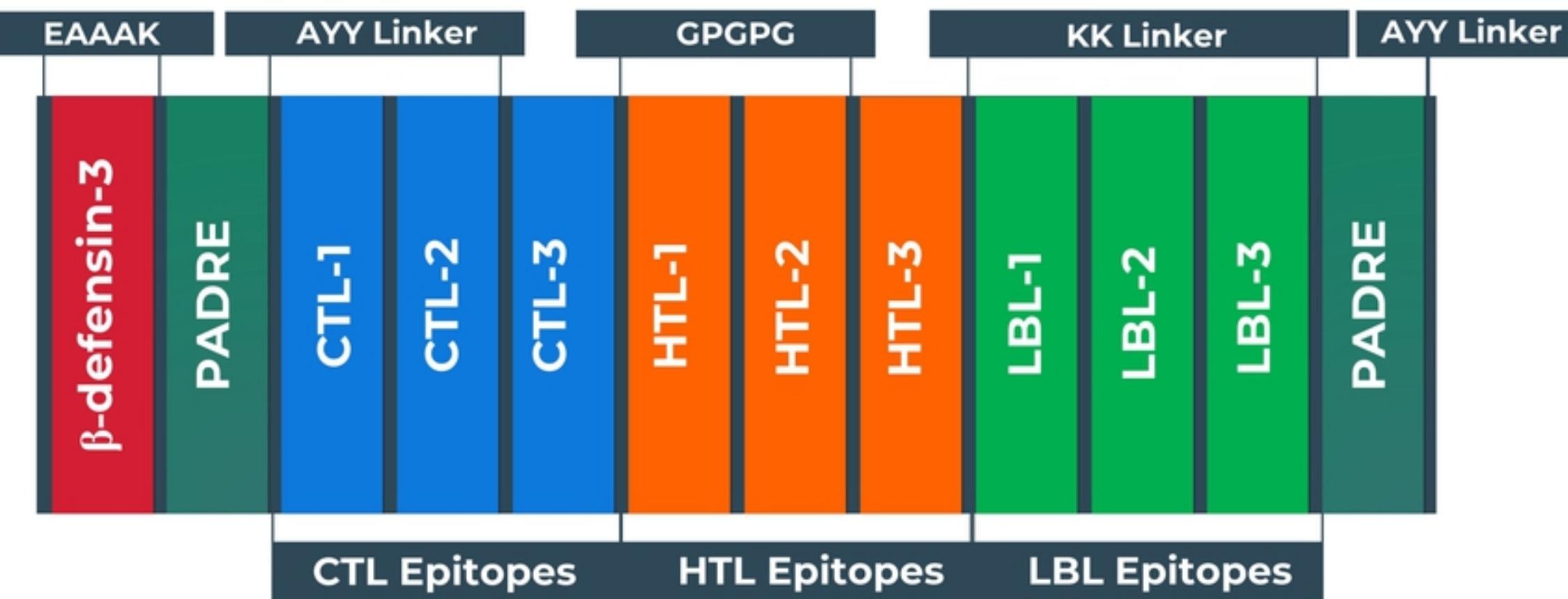
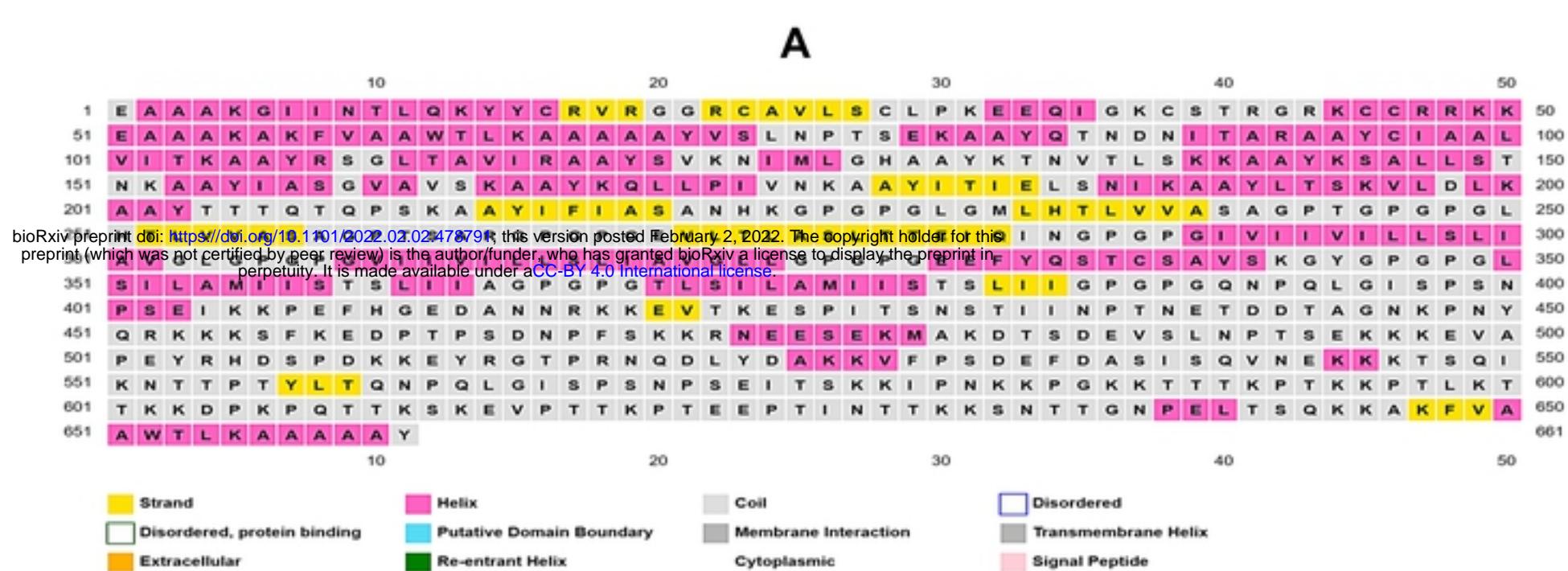


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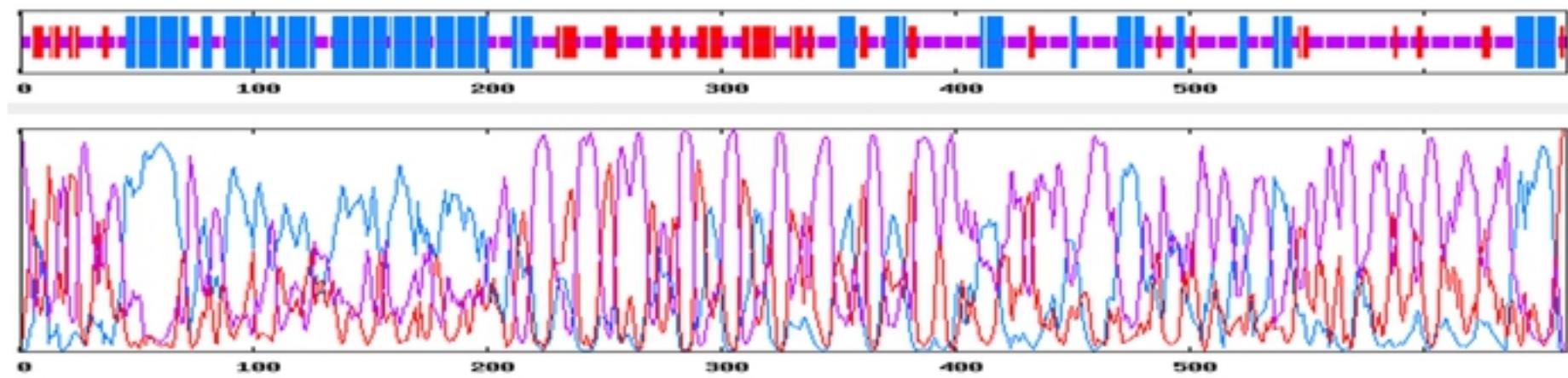
**A****B**

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 RSGLTAVIRAAYSVKNIMLGHAAAYKTNVTLSKKAAYKSALLSTNKAAYIASGV  
 AVSKAAAYKQLLPIVNKAAYITIELSNIKAAAYLTSKVLDLKAAYTTTQTQPSKAAY  
 IFIASANHK**GPGPGLGMLHTLVVASAGPTGPGPGLHTLVVASAGPTSARGPGP**  
**GEVLTLASLTTEIQINGPGPGIVIIVILLSLIAVGLGPGPVIIVILLSLIAVGLLGP**  
**GPGEEFYQSTCSAVSKGYGPGPGLSILAMIISTSLIIAGPGPGTLSILAMIISTSLII**  
**GPGPGQNPQLGISPSNPSEIKKPEFHGEDANNRKKEVTKESPITSNSTIINPTNET**  
 DDTAGNKP NYQRKKKSFKEDPTPSDNPFS**KKRNEESEKMAKDTSD**EVS LNPTSE  
 KKKEVAPEYRHDSPD**KKEYRGTPRNQDLYDAKKV**FPSDEFDASISQVNEKKKT  
 SQIKNTTPTYL TQNPQLGISPSNPSEITS**KKIPNKKPGKTTKPTK**PTLKKKD  
 PKPQTTKSKEVPTTKPTEEPTINT**KKSNTTG**NPELTSQ**KKAKFVAAWTL**KA  
**AAAY**

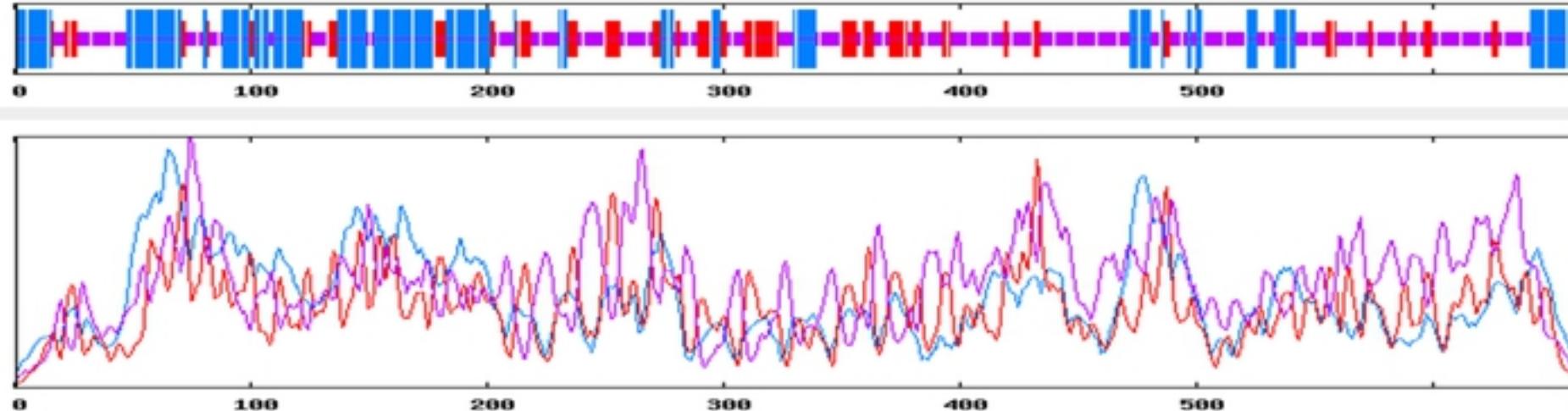
**Fig 3**



**B**



**C**



**D**

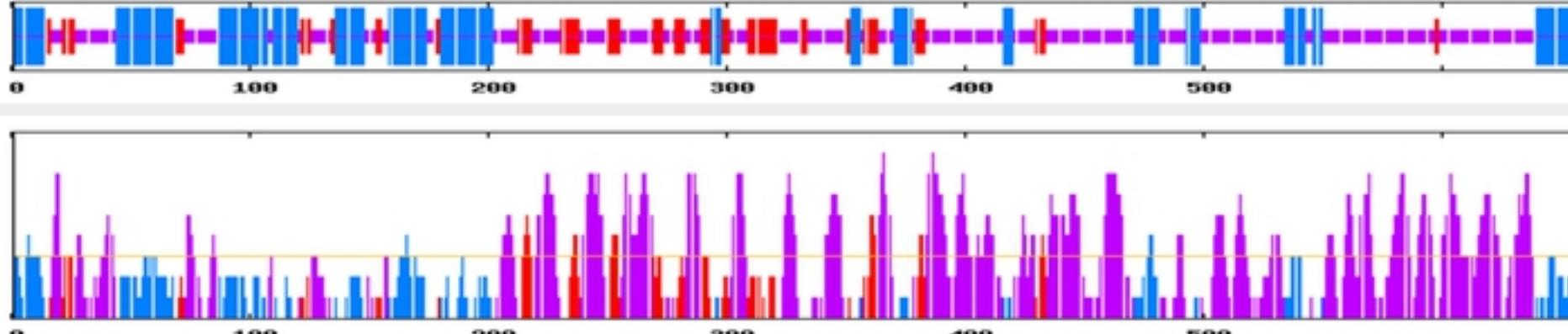
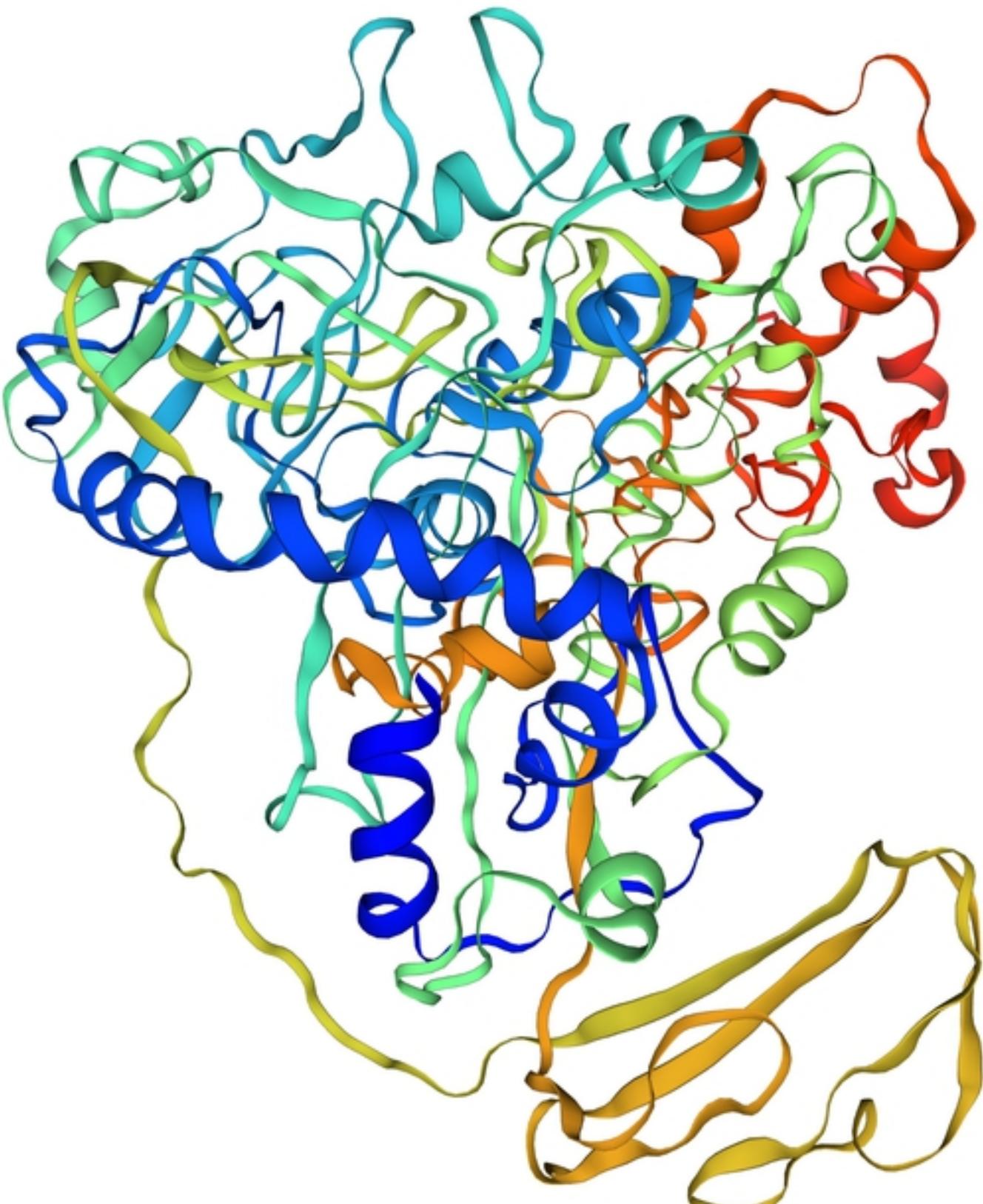
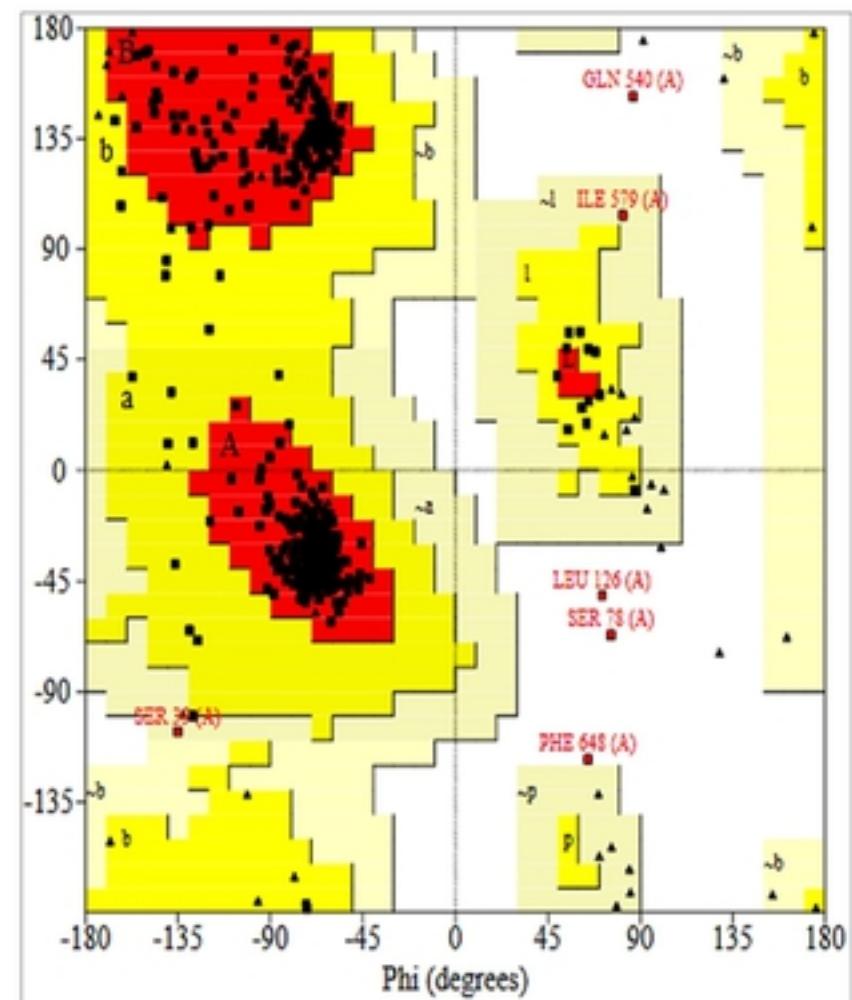


Fig 4

A



B



C

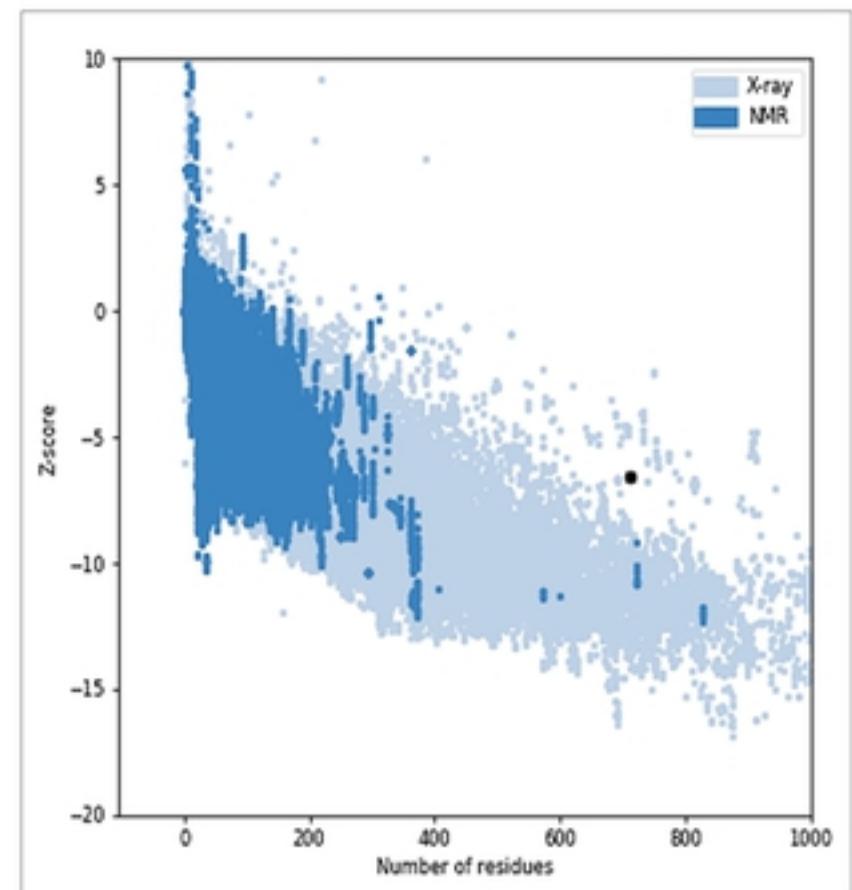


Fig 5

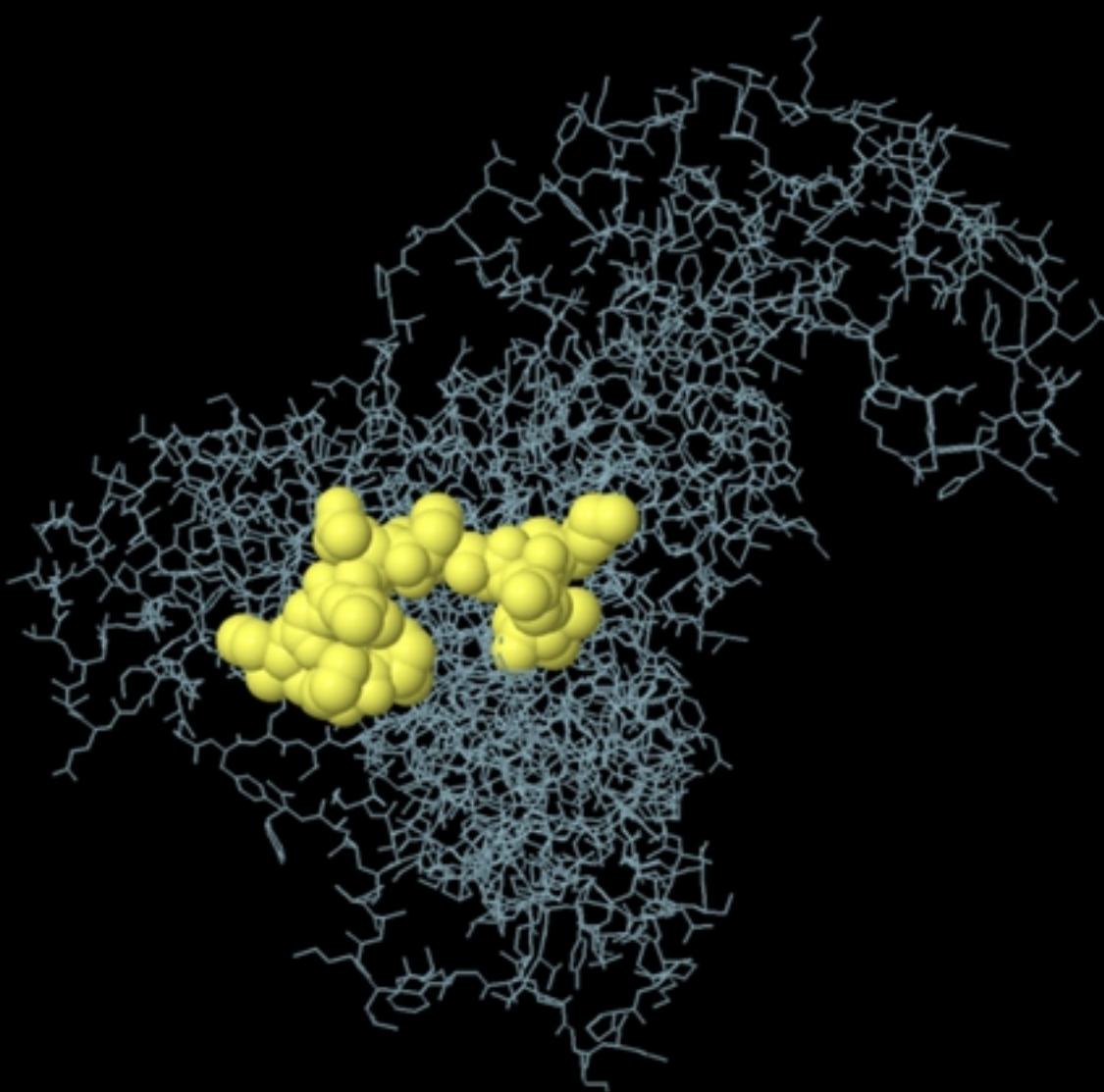
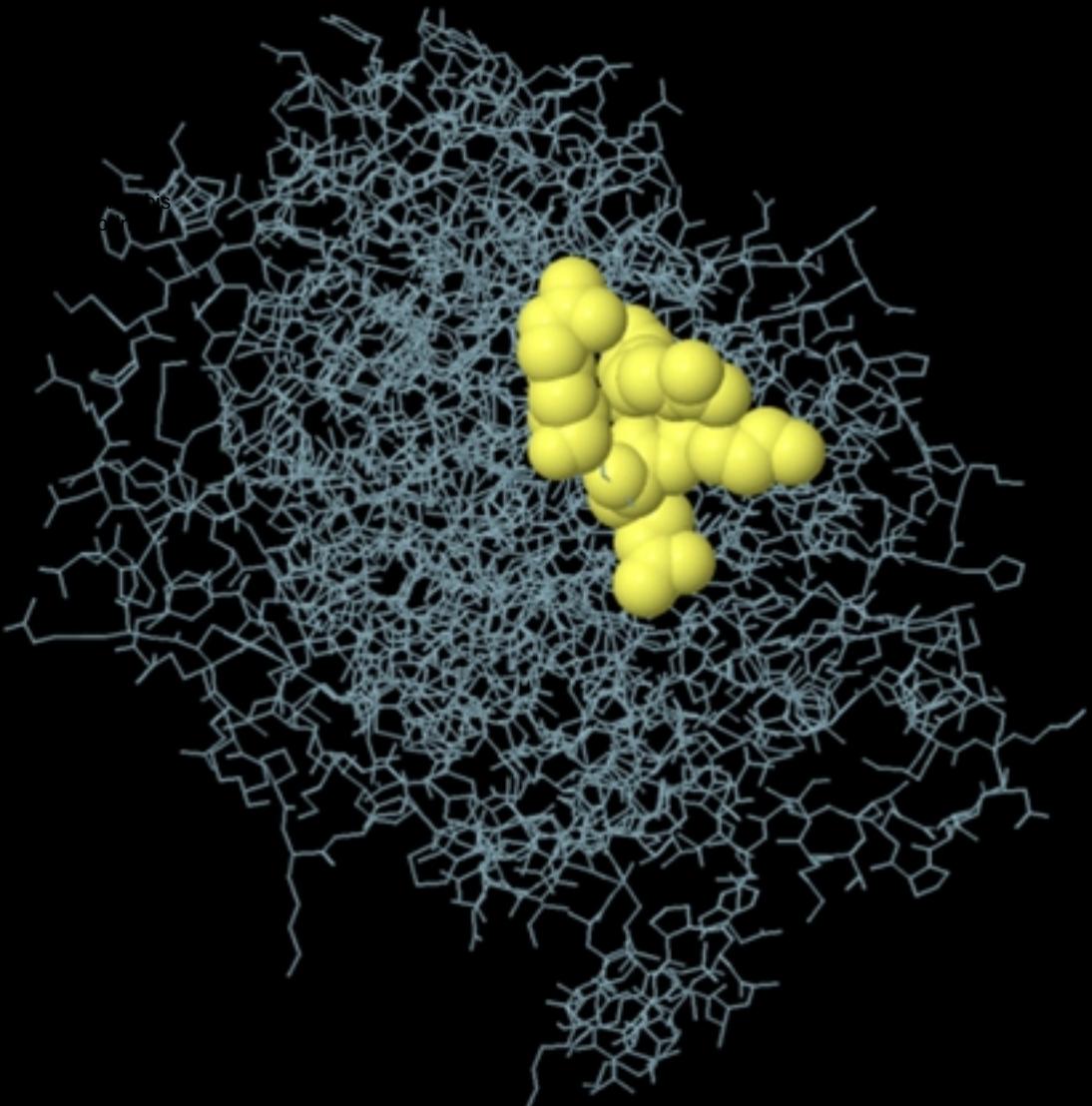
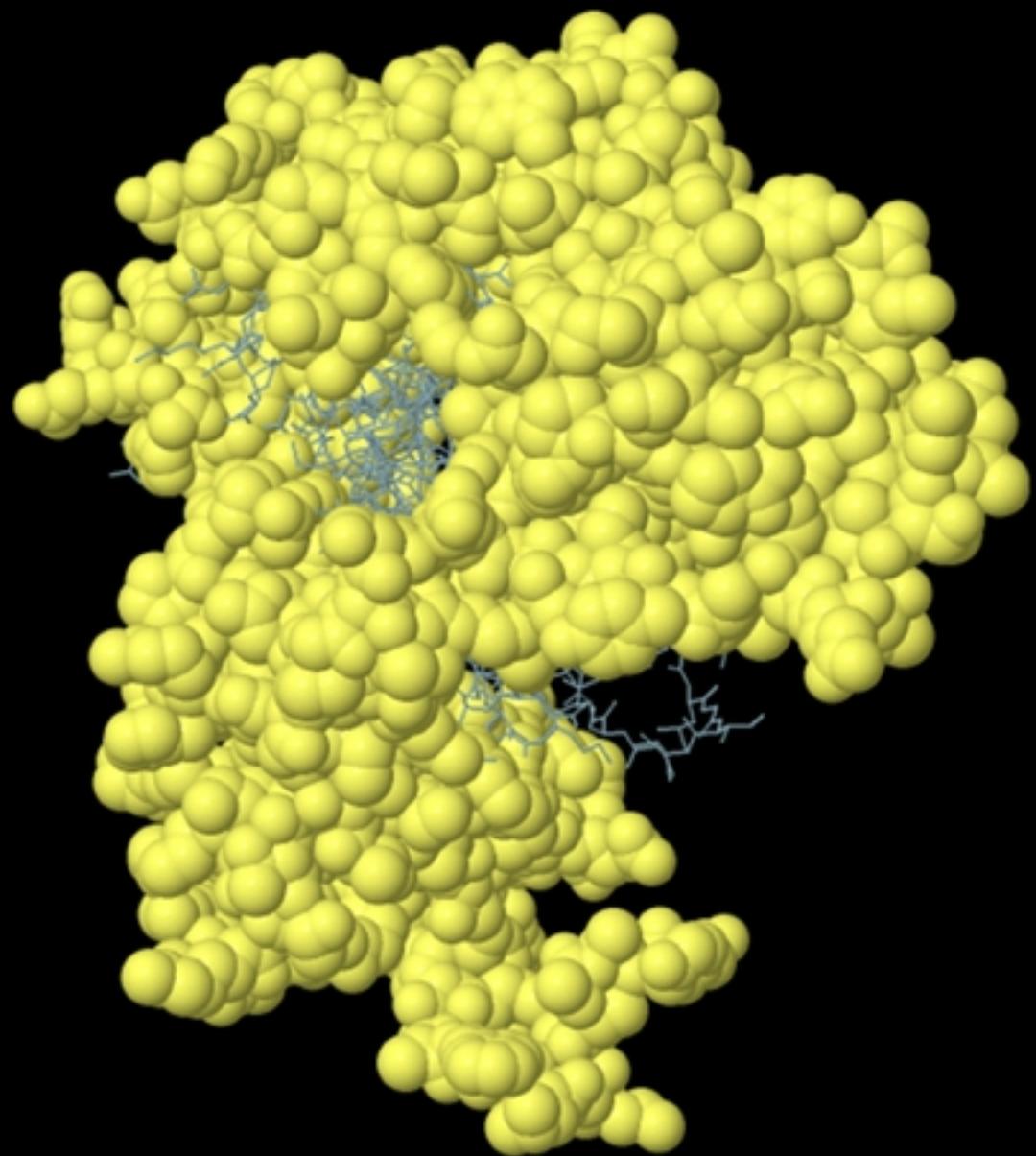
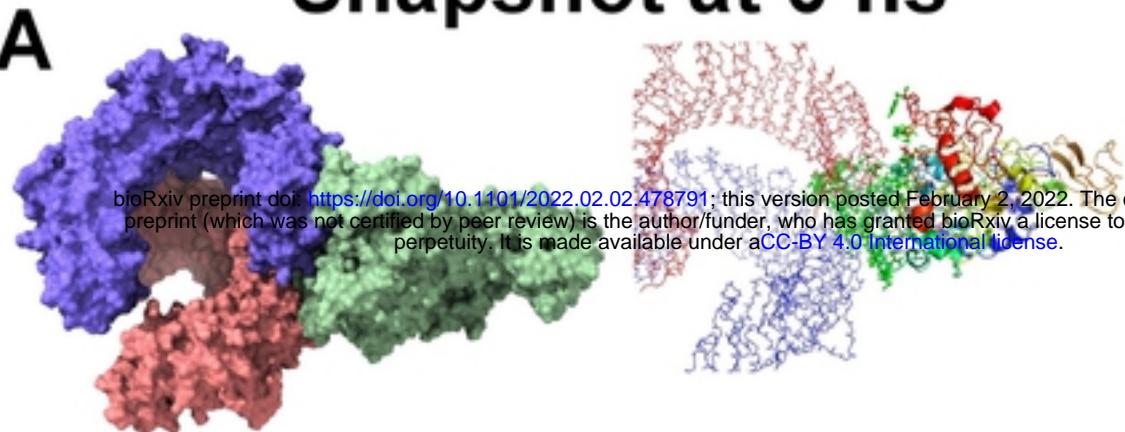
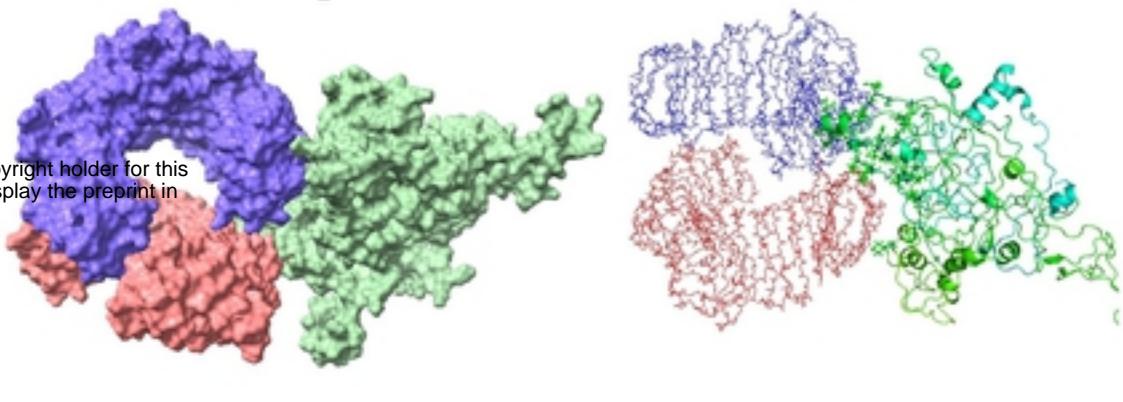


Fig 6

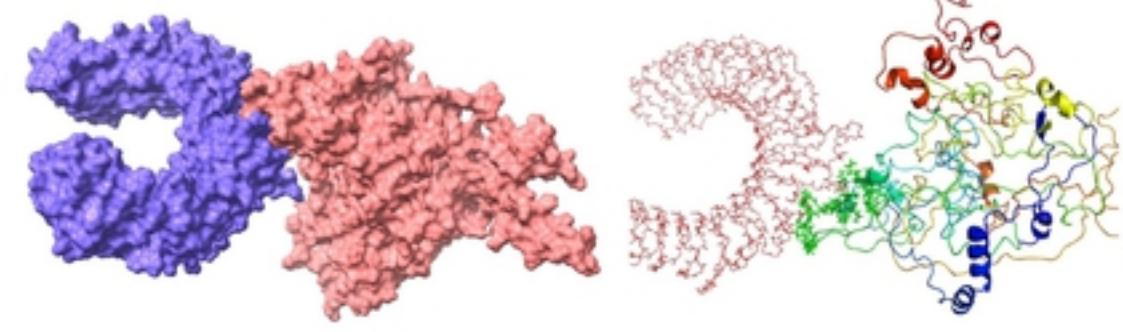
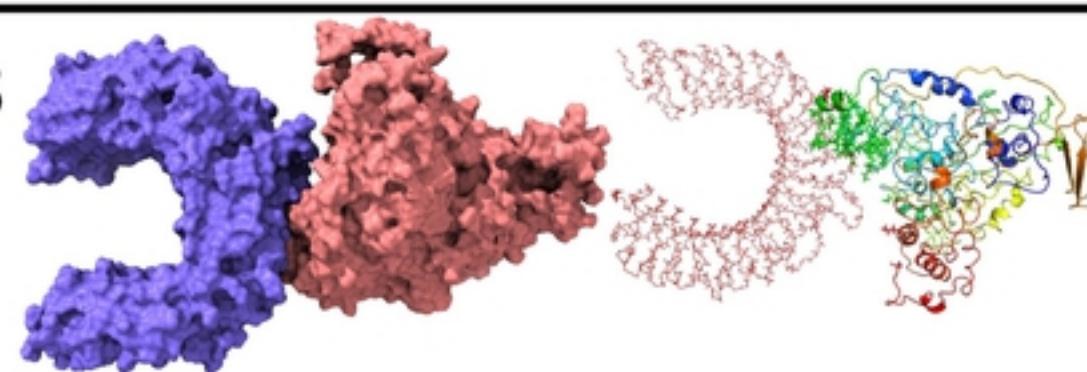
## Snapshot at 0 ns



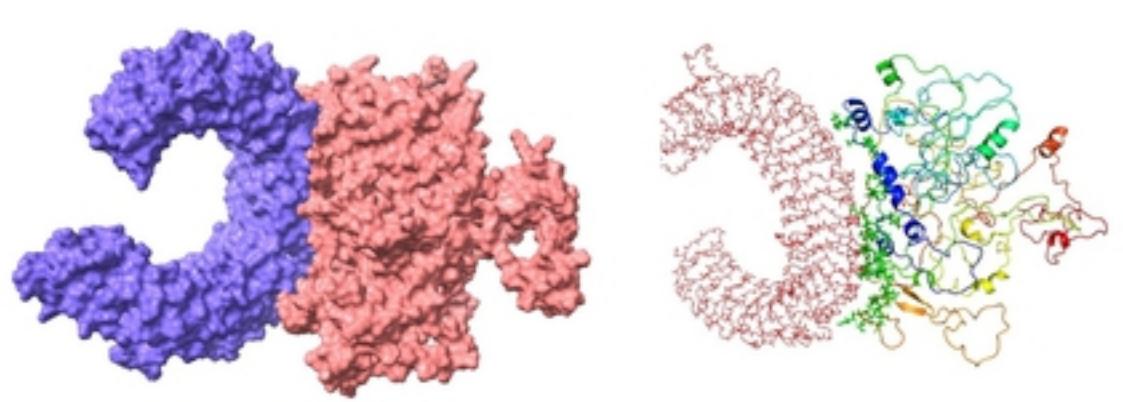
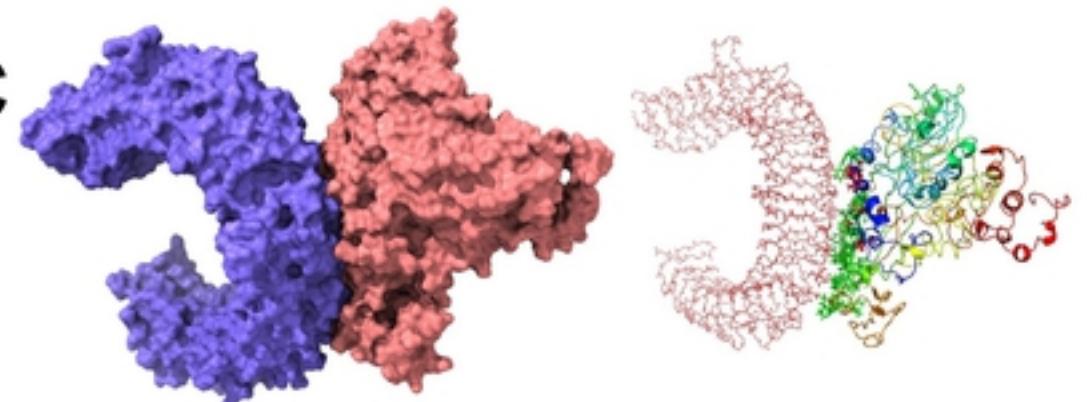
## Snapshot at 100 ns



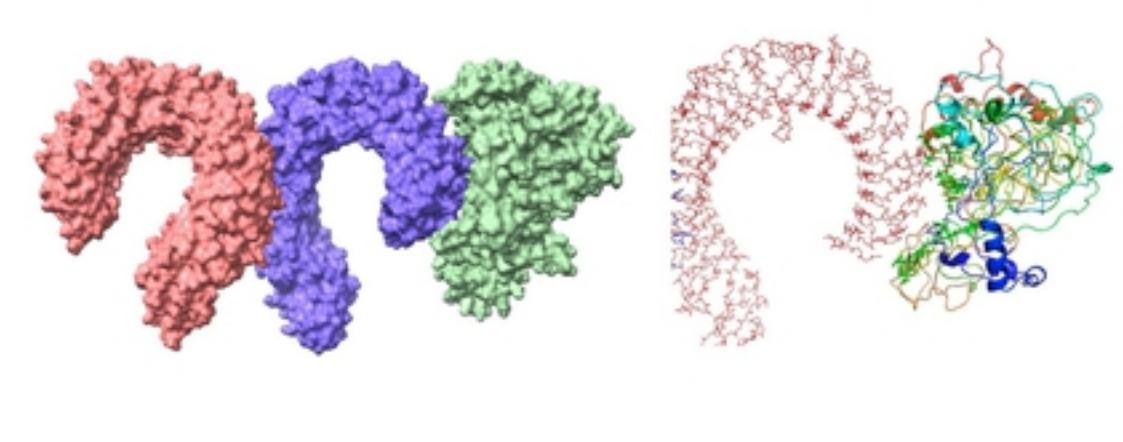
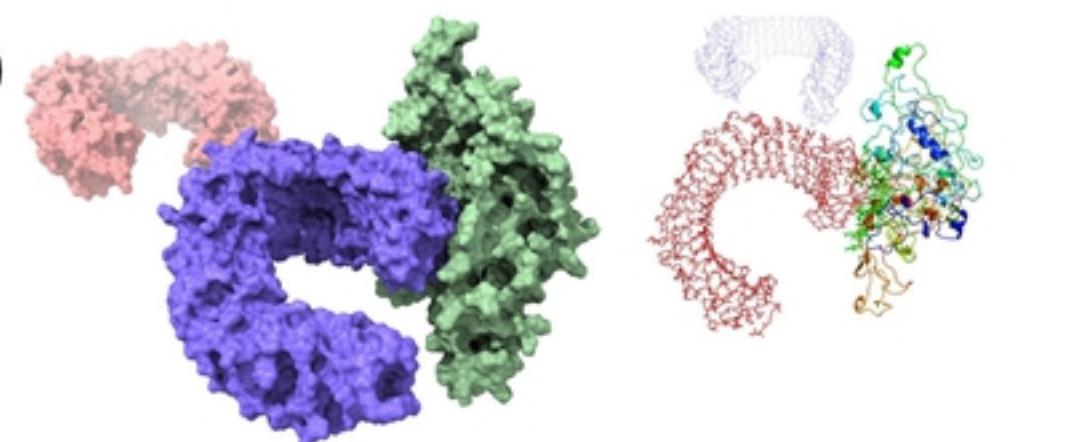
**B**



**C**



**D**



**E**

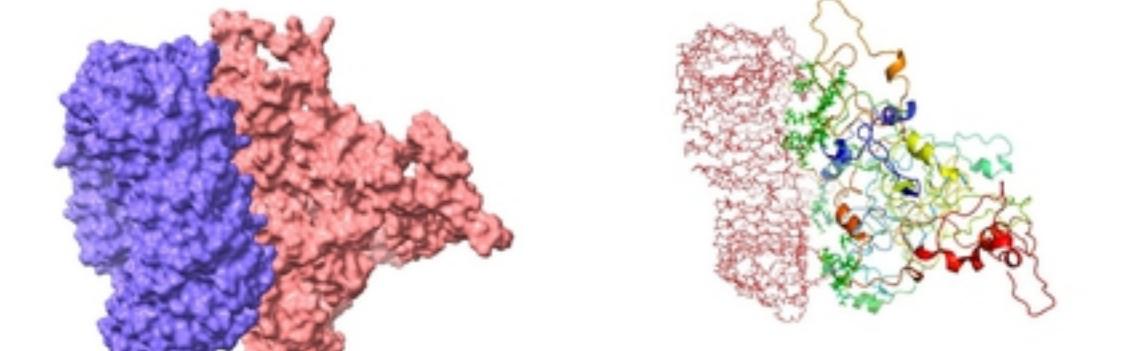
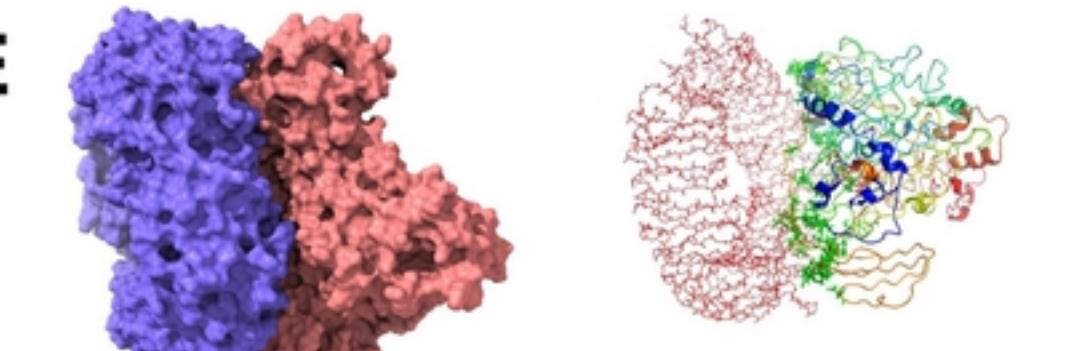


Fig 7

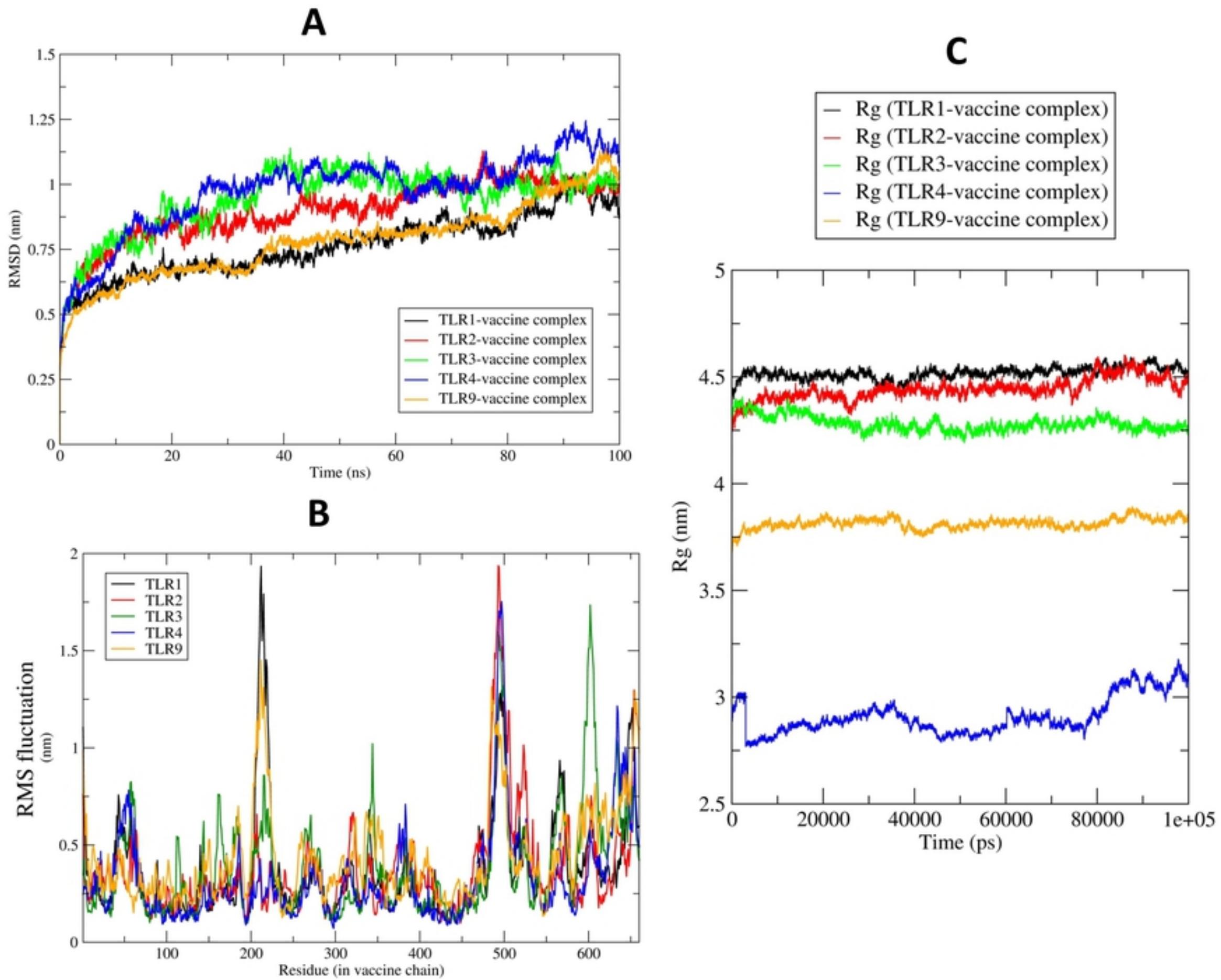


Fig 8

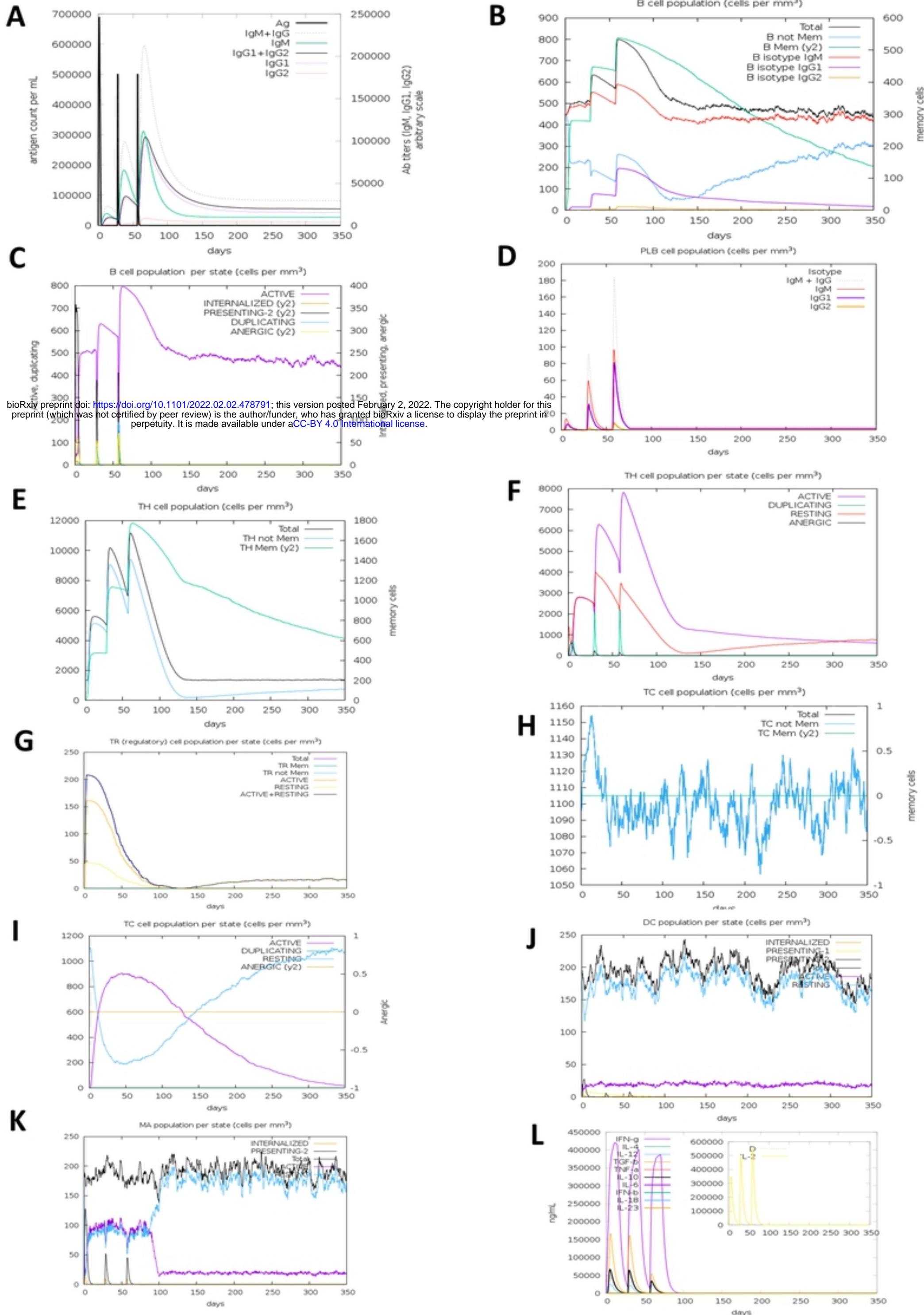


Fig 9