Chapter 26

Epitope Mapping by Epitope Excision, Hydrogen/Deuterium Exchange, and Peptide-Panning Techniques Combined with In Silico Analysis

Nicola Clementi, Nicasio Mancini, Elena Criscuolo, Francesca Cappelletti, Massimo Clementi, and Roberto Burioni

Abstract

The fine characterization of protective B cell epitopes plays a pivotal role in the development of novel vaccines. The development of epitope-based vaccines, in fact, cannot be possible without a clear definition of the antigenic regions involved in the binding between the protective antibody (Ab) and its molecular target. To achieve this result, different epitope-mapping approaches have been widely described (Clementi et al. Drug Discov Today 18(9–10):464–471, 2013). Nowadays, the best way to characterize an Ab bound region is still the resolution of Ab–antigen (Ag) co-crystal structure. Unfortunately, the crystallization approaches are not always feasible. However, different experimental strategies aimed to predict Ab–Ag interaction and followed by in silico analysis of the results may be good surrogate approaches to achieve this result. Here, we review few experimental techniques followed by the use of "basic" informatics tools for the analysis of the results.

Key words B cell epitope, Epitope excision, Hydrogen/deuterium exchange, Peptide-panning techniques, Epitope-based vaccines

1 Introduction

The in silico study of the Ab–Ag interaction spans from the prediction of their docking through methods based solely on computational analysis to the three-dimensional (3D) rendering of the Ag regions involved in the binding. While the first approach requires the use of different scoring functions and/or bioinformatic algorithms calculated by computational tools, the second one depends on the generation of experimental data to be further elaborated [1]. For the sake of brevity, here we describe only the latter approaches by reviewing three possible epitope-mapping strategies,

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combining empirical data generation and in silico analysis. Two of them (epitope excision, hydrogen/deuterium (H/D) exchange) are characterized by generation of putative epitope amino acid sequence data through mass spectrometry. The third approach (peptide panning) allows to obtain a putative epitope consensus sequence after affinity selection on the Ab of interest of a phage-bound peptide library. In both cases the data generated from these approaches are amino acid sequences to be analyzed in silico.

2 Materials

2.1 Epitope Excision

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

1. CN-Br Sepharose beads (GE Healthcare): Weigh 0.2 g of beads and rinse in 10 mL HCl 1 mM in a 15 mL centrifuge tube; shake for 15 min, and equilibrate for 15 min.
2. Filter column, provided with the CN-Br Sepharose beads.
3. HCl: 1 mM solution in water.
4. $NaHCO_3$: 100 mM solution in water, pH 8.3.
5. Coupling buffer: $NaHCO_3$ 100 mM, pH 8.3 and NaCl 500 mM.
6. Quenching buffer: Tris–HCl 100 mM, pH 8.0.
7. Washing buffer: Sodium acetate 100 mM, pH 4 and NaCl 500 mM.
8. Phosphate-buffered saline (PBS): Sodium phosphate buffer 100 mM, NaCl 150 mM, pH 7.2.
9. Bissulfosuccinimidyl suberate (BS3): 10 mM solution in PBS (*see* **Note 1**).
10. Working solution: NH_4HCO_3 50 mM, pH 7.8.
11. Formic acid: 0.1 % solution in water.
12. Complete protease inhibitor (Complete EDTA-free, Roche): Prepare the stock solution 25× according to the producer's instructions.
13. Antibody immobilized on the beads: Store in sodium phosphate 10 mM and NaCl 250 mM, pH 7.6; the optimal ratio is 1–10 μM of ligand for each mL of beads (*see* **Note 2**).
14. Antibody of interest: Prepare a solution of 50 μg of protein and 100 μL of coupling buffer; adjust with PBS to a final volume of 200 μL (*see* **Note 3**).
15. Antigen: Prepare a 200 μL solution with 50 μg of protein diluted in PBS, pH 7.2.

16. Trypsin stock solution: 100 μg/mL solution in HCl 1 mM.
17. Trypsin working solution: Dilute just before use the Trypsin stock solution in working solution; use an enzyme/protein ratio (w/w) between 1:20 and 1:100.

2.2 Hydrogen/ Deuterium Exchange

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

Important: Diligently follow all waste disposal regulations when disposing waste materials.

1. POROS 20 AL media (Applied Biosystems).
2. Sodium cyanogenborohydride ($NaCNBH_3$).
3. Saturated sodium sulfate (Na_2SO_4) solution.
4. PBS: NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, and KH_2PO_4 1.8 mM, pH 7.4.
5. Capping buffer: 2 mL of PBS containing ethanolamine 1 M and 8 mg/mL of $NaCNBH_3$.
6. Deuterated buffer: Phosphate 10 mM and NaCl 200 mM, pH 7.2 in 90 % D_2O.
7. Aqueous buffer: Phosphate 10 mM and NaCl 200 mM, pH 7.2.
8. Formic acid: 0.8 % solution in water.
9. Quenched solution: Guanidine hydrochloride (GuHCl) 1.6 M, 0.8 % formic acid.
10. Pepsin column (Hamuro Y, Coales SJ, Molnar KS, Tuske SJ, Morrow JA. *Rapid Commun. Mass Spectrom. 2008*; 22: 1041).
11. Trifluoroacetic acid (TFA): 0.05 % solution in water.
12. Reversed-phase trap column (4 μL bed volume; Applied Biosystems).
13. C18 column (Michrom BioResources, Inc.).
14. Solvent A: 0.05 % TFA solution in water.
15. Solvent B: 95 % acetonitrile and 5 % buffer A.
16. Antibody solution: Dilute in its buffer to a final concentration of 4.4 mg/mL.
17. Antigen solution: Dilute in its buffer to a final concentration of 1 mg/mL.

2.3 Peptide Panning

1. Phage Display Peptide Library 12-mer 100 μL, ~1×10^{13} pfu/mL (NEB—*New England BioLabs, Inc.*).
2. 96 gIII sequencing primer: 5′-CCC TCA TAG TTA GCG TAA CG-3′.
3. 28 gIII sequencing primer: 5′-GTA TGG GAT TTT GCT AAA CAA C-3′.

4. *E. coli* ER2738 host strain—F′ proA + B + lacIq Δ(lacZ) M15 zzf::Tn10(TetR)/fhuA2 glnV Δ(lac-proAB) thi-1 Δ(hsdS-mcrB)5.
5. SB medium—per liter: 35 g Bacto-Tryptone, 20 g yeast extract, 5 g NaCl. Autoclave, and store at room temperature.
6. LB medium—per liter: 10 g Bacto-Tryptone, 5 g yeast extract, 10 g NaCl. Autoclave, and store at room temperature.
7. Isopropyl-β-D-thiogalactoside (IPTG)/5-Bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) stock—Mix 1.25 g IPTG and 1 g Xgal in 25 mL dimethyl formamide (DMF). Store at −20 °C.
8. LB/IPTG/Xgal plates—1 l LB medium + 15 g/L agar. Autoclave, cool to <70 °C, add 1 mL IPTG/Xgal stock per liter, and pour. Store at 4 °C in the dark.
9. Top agar—per liter: 10 g Bacto-Tryptone, 5 g NaCl, 7 g Bacto-Agar.
10. Tetracycline stock (suspension)—20 mg/mL in 1:1 ethanol: water. Store at −20 °C.
11. LB + Tet plates—1 l LB medium + 15 g/L agar. Autoclave, cool to <70 °C, add 1 mL tetracycline stock, and pour. Store at 4 °C in the dark.
12. Blocking buffer—0.1 % PBS/BSA. Filter sterilize, and store at 4 °C.
13. PBS 1×. Autoclave, and store at room temperature.
14. PEG/NaCl—20 % (w/v) polyethylene glycol—8000, 2.5 M NaCl. Autoclave, and store at room temperature.
15. Washing solution—PBS 1× + 0.1 % [v/v] Tween-20.
16. Elution buffer—0.1 M HCl pH 2.2. Filter sterilize, and store at 4 °C.
17. Neutralizing buffer—1 M Tris base, pH 9. Filter sterilize, and store at 4 °C.

3 Methods

3.1 Epitope Excision

This epitope-mapping technique has been developed considering the differential accessibility of surface-exposed amino acid residues of the antigen in the presence or the absence of the antibody of interest [2]. Briefly, the Ab to be characterized is incubated with its target and digested. The epitope and the paratope complex will not be digested, and the amino acid composition of the eluted epitope will be evaluated through mass spectrometry by subtracting the unbound Ag control and the unbound Ab as well (Fig. 1).

Carry out all procedures at room temperature unless otherwise specified.

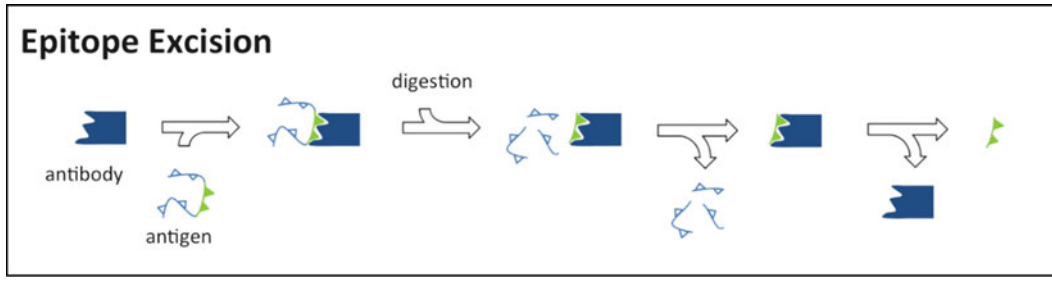

Fig. 1 Schematic representation of the experimental procedure of the Epitope Excision protocol: the antigen is bound to the immobilized antibody and digested with endoproteinases. Unbound peptides are washed off, and the affinity-bound peptides are subsequently eluted and collected to be further analyzed

Important: Opportune controls must be included in order to perform correctly the further mass spectrometric analysis since the exclusion of peptides obtained from the digestion of the unbound Ag or the unbound Ab of interest is essential.

For this purpose we suggest to perform the following digestions (a and b) in order to compare the differences between "a, b" resulting data and those obtained from the mAb-bound antigen (all the digestions must be performed in column).

(a) Digestion on antigen → add the Ag to a dedicated column without Ab bound to the beads.

(b) Digestion on the antibody → digest the Ab bound to the beads, without the presence of the antigen.

1. Rinse the CN-Br Sepharose beads as described, and take 200 µL of the slurry from the bottom of the centrifuge tube; place it in a 1.5 mL collection tube and spin down in a tabletop microcentrifuge.
2. Wash 6× with HCl solution: Add 800 µL each time, centrifuge in a tabletop microcentrifuge at 13,000 RCF for 1 min, and discard the supernatant.
3. Wash 6× with NaHCO3 solution, 400 µL each time as described above.
4. Centrifuge one more time at 13,000 RCF for 1 min, and discard the supernatant.
5. Add 100 µL of coupling buffer with the desired amount of antibody to be immobilized on the beads, and check that the pH value of the solution of beads and protein has not been altered.
6. Incubate for 2 h with slow agitation.
7. Centrifuge at 13,000 RCF for 1 min, and discard the supernatant.
8. Wash with 400 µL quenching buffer as described above.

9. Incubate for 2 h with 400 µL quenching buffer (*see* **Note 4**).
10. Wash 6× with alternating washing buffer and quenching buffer, 400 µL each time as described above.
11. Add the antibody of interest and incubate overnight (o.n.) at 4 °C (*see* **Note 5**).
12. Centrifuge at 13,000 RCF for 1 min, and discard the supernatant.
13. Wash 3× with PBS, 400 µL each time as described above.
14. Add 100 µL of BS3 to cross-link the antibody immobilized on the beads to the antibody of interest.
15. Incubate for 45 min with slow agitation.
16. Centrifuge at 13,000 × *g* for 1 min, and discard the supernatant.
17. Wash 2× with quenching buffer, 400 µL each time as described above.
18. Wash 3× with PBS, 400 µL each time as described above.
19. Add the antigen solution and incubate for 2 h with slow agitation.
20. Centrifuge at 13,000 RCF for 1 min, and discard the supernatant.
21. Wash 3× with PBS, 400 µL each time as described above.
22. Centrifuge at 13,000 RCF for 1 min, and discard the supernatant.
23. Add 100 µL of trypsin working solution and incubate o.n. at 37 °C.
24. Add the slurry on the filter column provided and centrifuge at 13,000 RCF for 1 min.
25. Collect the flow through, and add 4 µL of complete protease inhibitor stock solution.
26. Wash the column 3× with PBS, 400 µL each time as described above.
27. Add 500 µL of formic acid and incubate for 15 min with slow agitation.
28. Centrifuge at 13,000 RCF for 1 min, and collect the elution.
29. Store at 4 °C.
30. Follow proper sample preparation techniques depending on the mass spectrometric approach selected (MALDI-MS or ESI-MS) for further analysis.

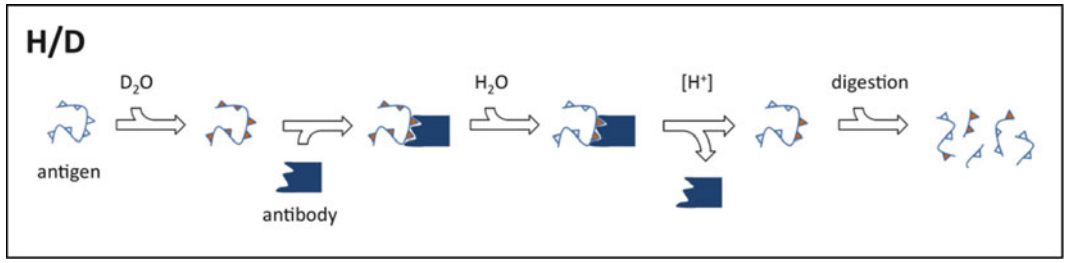

Fig. 2 The on/off-exchange protocol: Schematic representation of the experimental procedure. The antigen, firstly treated with a deuterated solution, is bound to the immobilized antibody. The complex is washed with a water solution allowing the ion exchange only on the unbound and exposed surfaces. Subsequently, the antigen is eluted and digested with endoproteinase. Finally, the MS measurement permits the identification of deuterated peptides

3.2 Hydrogen/ Deuterium Exchange

Here is described the "on-solution/off-column" (on-exchange in solution followed by off-exchange in column) experiment where the antigen is first mixed with a deuterated buffer in solution and incubated for a predetermined duration ("on-solution" = on-exchange in solution) [3]. The on-exchanged antigen is next loaded onto the antibody column. The antigen-bound column is then washed with aqueous buffer allowing the ion exchange only on the unbound and exposed surfaces and incubated for half of the solution on-exchange time ("off-column" = off-exchange in column; the intrinsic D → H exchange reaction is twice as fast as the intrinsic H → D exchange reaction at the same pH reading due to isotopic effects). After the on/off-exchange reaction, the antigen is eluted out by a low pH buffer and digested by pepsin. Finally, the MS measurement permits the identification of deuterated peptides (Fig. 2).

Carry out all procedures at room temperature unless otherwise specified.

Important: A fully deuterated sample and a non-deuterated sample must be included in the experiment in order to fulfil the determination of deuteration level of each peptide after the on/off-exchange reaction.

3.2.1 Immobilization of the Antibody

1. Add 100 mg of POROS AL and 5 mg $NaCNBH_3$ to 600 μL of antibody solution.
2. Add 800 μL of saturated sodium sulfate (Na_2SO_4) solution and incubate *o.n.* with agitation.
3. Wash 5× with PBS.
4. Add the capping buffer and incubate for 2 h with slow agitation.

   (*Note*: This step is required in order to cap the unreacted aldehyde groups.)
5. Wash 5× with PBS, and resuspend in PBS.
6. Pack the resin in a column with 353 μL bed volume.

3.2.2 "On-Solution/ Off-Column" Experiment

1. Dilute 5 μL of antigen solution with 45 μL of deuterated buffer.
2. Incubate at 38 °C for varying times (150, 500, 1,500, and 5,000 s).
3. Load the on-exchanged solution onto the antibody column (pre-equilibrated with deuterated buffer).
4. Wash the column with 500 μL of aqueous buffer.
5. Incubate for one-half of the preceding on-exchange duration (75, 250, 750, and 2,500 s).
6. Add 320 μL of formic acid (*see* **Note 6**).
7. Collect the last 40 μL of the eluent.
8. Add 20 μL of quenched solution.

3.2.3 Fully Deuterated Experiment

1. Add 4 μL of antigen solution to 36 μL of deuterated buffer and incubate at 60 °C *o.n.*
2. Load the sample onto the antibody column (pre-equilibrated with deuterated buffer).
3. Wash with 100 μL of deuterated buffer.
4. Add 320 μL of formic acid.
5. Collect the last 40 μL of the eluent.
6. Add 20 μL of quenched solution.

3.2.4 General Process for H/D-Exchanged Sample

1. Pump the quenched solution over a pepsin column (104 μL bed volume) with TFA (200 μL/min) for 3 min.
2. At the same time collect the proteolytic products by a reversed-phase trap column (4 μL bed volume).
3. Elute the peptide fragments and separate using a C18 column with a linear gradient of 13 % solvent B to 40 % solvent B over 23 min (flow rate 10 to 5 μL/min).
4. Follow proper sample preparation techniques depending on the mass spectrometric approach selected (MALDI-IRMPD or FI-TICR MS) for further analysis.

3.3 Peptide Panning

This epitope-mapping approach is based on the affinity selection of phage-displayed peptides against the Ab of interest (Fig. 3) [4–7]. Interestingly, this approach can lead to identification of mimotopes to be used in the rational design of epitope-based vaccine approaches [8]. This method can be followed using different phage libraries, spanning from commercially available libraries (containing linear peptides as well as loop-constrained peptides) to libraries deriving from the enzymatic digestion of the antigen of interest. As an example, we describe a "standard" panning procedure adapted from the screening protocol of Peptide Library 12-mer indicated by *New England BioLabs Inc.*

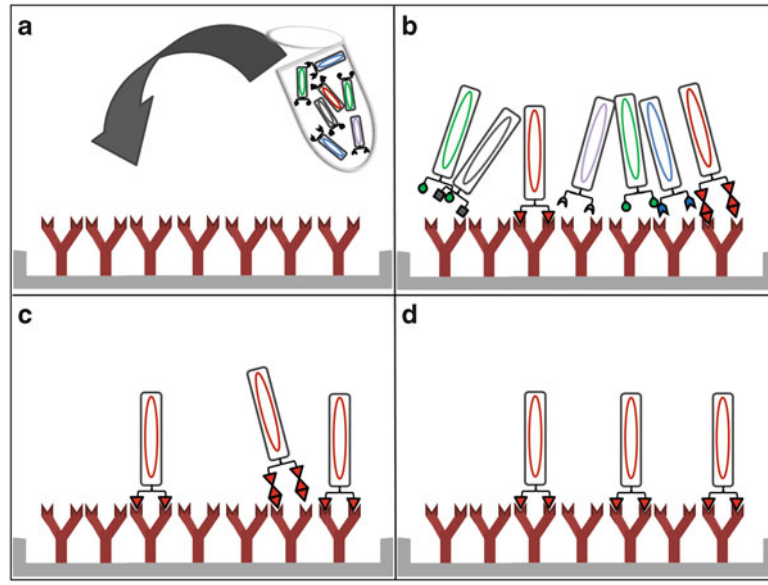

Fig. 3 Schematic representation of peptide panning. (**a**) A peptide phage library (commercially available or "homemade") is "panned" with the Ab to be characterized coated on a flat-bottom plate. (**b**) The library is then incubated at 37 °C. (**c**) Unbound phages are washed away. (**d**)After selection rounds, single high-affinity clones are picked up and sequenced

3.3.1 Phage Titering

1. Inoculate 5–10 mL of SB with ER2738 (single colony) and incubate at 37 °C with shaking, for 4–8 h. When the culture reaches mid-log phase, dispense 200 μL into microfuge tubes.
2. While cells are growing, melt top agar in microwave and dispense 3 mL into sterile culture tubes, one per expected phage dilution. Maintain tubes at 45 °C.
3. Prepare serial dilutions of phage in SB. Suggested dilution ranges: for amplified phage culture supernatants, 10^{-8} to 10^{-11}; for unamplified panning eluates, 10^{-1} to 10^{-4}.
4. Add 1 μL of each phage dilution to each tube to carry out infection, vortex quickly, and incubate for 15 min at 37 °C.
5. Transfer the infected cells to culture tubes containing 45 °C top agar. Shake and pour culture onto a pre-warmed LB/IPTG/Xgal plate. Tilt gently and rotate plate to spread top agar.
6. Allow the plates to cool for 5 min at room temperature and incubate overnight at 37 °C.
7. Phage plaques to be counted will appear in blue.

3.3.2 Peptide Panning

Day 1

1. Prepare Ab/PBS 1× solution (generally 300 ng of Ab per well), and coat four flat-bottom wells for each Ab to be characterized.
2. Add 25 μL of Ab-containing solution to each well and swirl gently until the well surfaces are completely covered by the coating solution.
3. Incubate overnight at 4 °C.

Day 2

4. Inoculate 10 mL of SB+Tet medium with a single colony of ER2738. This culture will be used for phage tittering (*see* **Note 7**).
5. Pour off the coating solution from each plate, and firmly slap it face down onto a clean paper towel to remove residual solution. Fill each plate or well completely with blocking buffer. Incubate for at least 1 h at 37 °C.
6. Discard the blocking solution as in **step 5**. Wash each plate rapidly six times with washing solution.
7. Dilute 100-fold a representation of the library with 40 μL of PBS/well. Pipette onto coated plate, and incubate at 37 °C for 2 h.
8. Discard nonbinding phage by pouring off and slapping plate face down onto a clean paper towel.
9. Wash plates ten times with the washing solution (**step 6**).
10. Elute bound phage with 50 μL of elution buffer. Rock gently for 10 min, scrape the wells, pipette eluate into a microcentrifuge tube, and neutralize with 3 μL/well of neutralizing buffer.
11. Titer the eluate generally using 1 μL diluted as described above. Plaques from the first- or second-round eluate titering can be sequenced if desired.
12. Inoculate 1 mL from the ER2738 culture performed at **step 4** in 100 mL SB. Add the eluate phages and incubate for 4.5 h at 37 °C with vigorous shaking.
13. Transfer the culture to a centrifuge tube (50 mL) and spin for 30 min at 4,000 × *g* at 4 °C. Transfer the supernatant to a fresh tube and respin (discard the pellet).
14. Transfer the upper 80 % of the supernatant to a fresh tube, and add to it 1/6 volume of 20 % PEG/2.5 M NaCl. Allow the phage to precipitate at 4 °C for at least 2 h, preferably overnight.

Day 3

15. Spin the PEG precipitation at 12,000 × *g* for 20 min at 4 °C. Decant and discard the supernatant, respin the tube briefly, and remove residual supernatant with a pipette.
16. Suspend the pellet in 1 mL of PBS. Transfer the suspension to a microcentrifuge tube and spin at maximum (18,000 × *g*) for 5 min at 4 °C to pellet residual cells.

17. Transfer the supernatant to a fresh microcentrifuge tube and reprecipitate by adding 1/6 volume of 20 % PEG/2.5 M NaCl. Incubate on ice for 60 min. Microcentrifuge at 18,000 RCF for 10 min at 4 °C, discard the supernatant, respin briefly, and remove residual supernatant with a micropipet.
18. Suspend the pellet in 1 mL of PBS. Microcentrifuge for 1 min to pellet any remaining insoluble material. Transfer the supernatant to a fresh tube. This is the amplified eluate.
19. Titer the amplified eluate as described above, on LB/IPTG/Xgal plates.
20. Coat the plate for the second round of panning as described in **steps 1–3**.

Days 4 and 5

21. Count blue plaques from the titering plates (**step 19**), and determine the phage titer, which should be on the order of $10^{13/14}$ pfu/ml. Succeeding rounds of panning can be carried out with as little as 10^9 pfu of input phage.
22. Carry out a second round of panning: Repeat **steps 4–18** using the first-round amplified eluate as input phage and raising the Tween concentration in the wash steps to 0.5 % (v/v).
23. Titer the resulting second-round amplified eluate on LB/IPTG/Xgal plates.
24. Coat a plate or a well for the third round of panning as described above.

Day 6

25. Carry out a third round of panning.
26. Titer the unamplified third-round eluate as in **step 11** on LB/IPTG/Xgal plates. Plaques from this titering can be used for sequencing.

    If there is no clear sequence consensus, carry out a fourth round of panning.

3.4 Data Analysis

The data of any epitope-mapping approach should be analyzed considering those previously obtained in the biological characterization of the mAb of interest, following "standard" experimental procedures (e.g., assays evaluating the Ab biological activity, western blotting, immunoprecipitation, Biacore). This will permit to focus better the data obtained from the three methods described above. The three epitope-mapping experimental approaches described above are just examples among epitope-mapping techniques [1, 7, 9–12]. We focused on these three approaches since the data resulting from all these methods are amino acidic sequences to be further analyzed in silico.

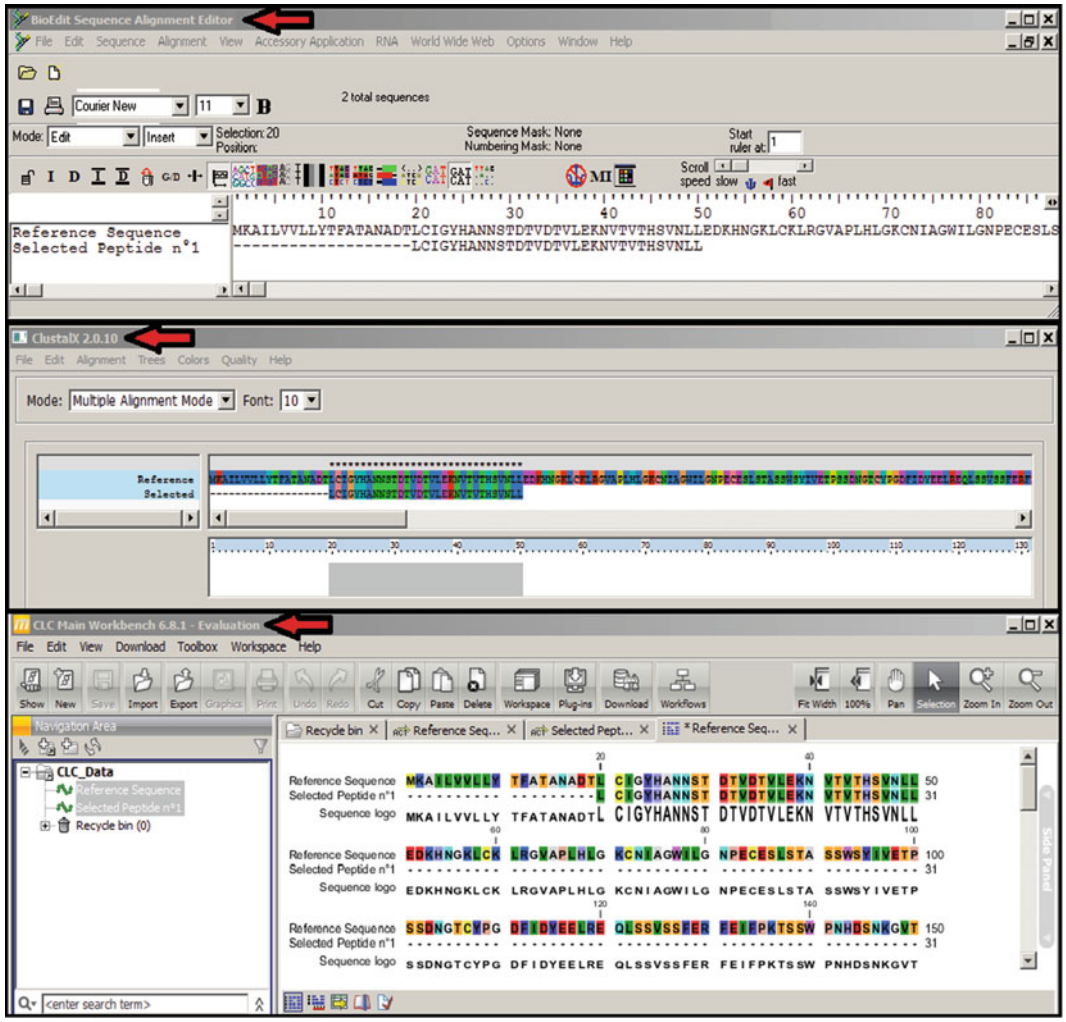

Fig. 4 Comparison between the reference sequence and the peptide sequence deriving from the two mass spectrometric approaches. *Red arrows* highlight three software commonly used for sequence analysis

3.4.1 Analysis of Data Deriving from the Mass Spectrometric Approaches

The amino acid sequences resulting from the mass spectrometric approaches described above should be analyzed by using dedicated database servers, software to "manage" the peptide sequences, and web servers or meta-web servers for the analysis of consensus motifs on the target antigen crystal structures (if available) [1]. In particular, the "custom" procedure should be performed as follows:

Sequence Alignment

The "output" sequence (sequence resulting from the experimental assays) must be checked by aligning it with a reference sequence (usually the amino acid sequence of the protein used to perform the assays). This step can be easily performed by using several programs such as ClustalX (http://www.clustal.org), Bio-Edit (http://www.mbio.ncsu.edu/bioedit/bioedit.html), or CLC-Workbench (http://www.clcbio.com) [13, 14] (Fig. 4). The same

result can also be achieved by uploading the sequence(s) (.fasta format) into Protein-Blast server (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). The latter allows for the discovery of sequence analogies among different antigenic "variants" of the same antigen (Fig. 5).

Once the correspondence between the reference linear amino acid sequence and the "output" sequence is checked, it is possible to identify (in the "reference" sequence) the antigenic region involved, i.e., in the Ab–Ag binding. Moreover, by highlighting the different protein functional and/or structural domains (when possible or if they are present) it is possible to evaluate potential consistencies between the epitope "position" in the linear sequence and an eventual biological activity peculiar of the Ab previously characterized (Fig. 6).

Identification of the Putative Epitope on 3D Crystal Structures or Molecule Models

When a crystal structure of the Ag is available, it can be helpful to identify the amino acid residues of the putative Ab-bound protein region on the 3D structure of the protein targeted by the Ab. When more than one structure are available, it can be important to use crystal structures endowed with the highest resolution. To do this, structures of the Ag of interest can be downloaded from Internet databases (e.g., RCSB Protein Data Bank, http://www.rcsb.org/pdb/home/home.do) (Fig. 7).

In order to visualize, edit, and analyze the downloaded crystal structures, it can be useful to use molecular visualization and editing computer programs freely available "online" such as UCSF Chimera, DeepView/SPDBV, VMD (more complex computer program designed for visualizing and analyzing molecular dynamics (MD) simulation and biological systems), RasMol, or Cn3D (Table 1).

Once the crystal structure (usually downloaded with .pdb extension) is loaded onto a rendering program (Table 1) (Fig. 8), the residues identified in the linear amino acid sequence can now be highlighted on the crystal (or model), allowing to appreciate the 3D conformational motifs of the putative Ab-bound region containing the epitope (Fig. 9).

The putative recognized motif, highlighted in the crystal structure, can now be further analyzed. In particular, it can be measured, evaluated for its hydrophilic or hydrophobic proprieties, and compared to epitopes belonging to Ab possibly well characterized, already described, and available for comparative experimental assays.

3.4.2 Analysis of the Affinity-Selected Peptides

The amino acid sequences of the peptide-panning selected peptides can be analyzed in order to find possible consensus motifs on the 3D structure of the Ab-targeted Ag. Also in this case, prior to proceeding with further analysis, the antigen crystal structure (or model) must be downloaded from dedicated protein structure databases (see above). Nowadays, different informatics tools able

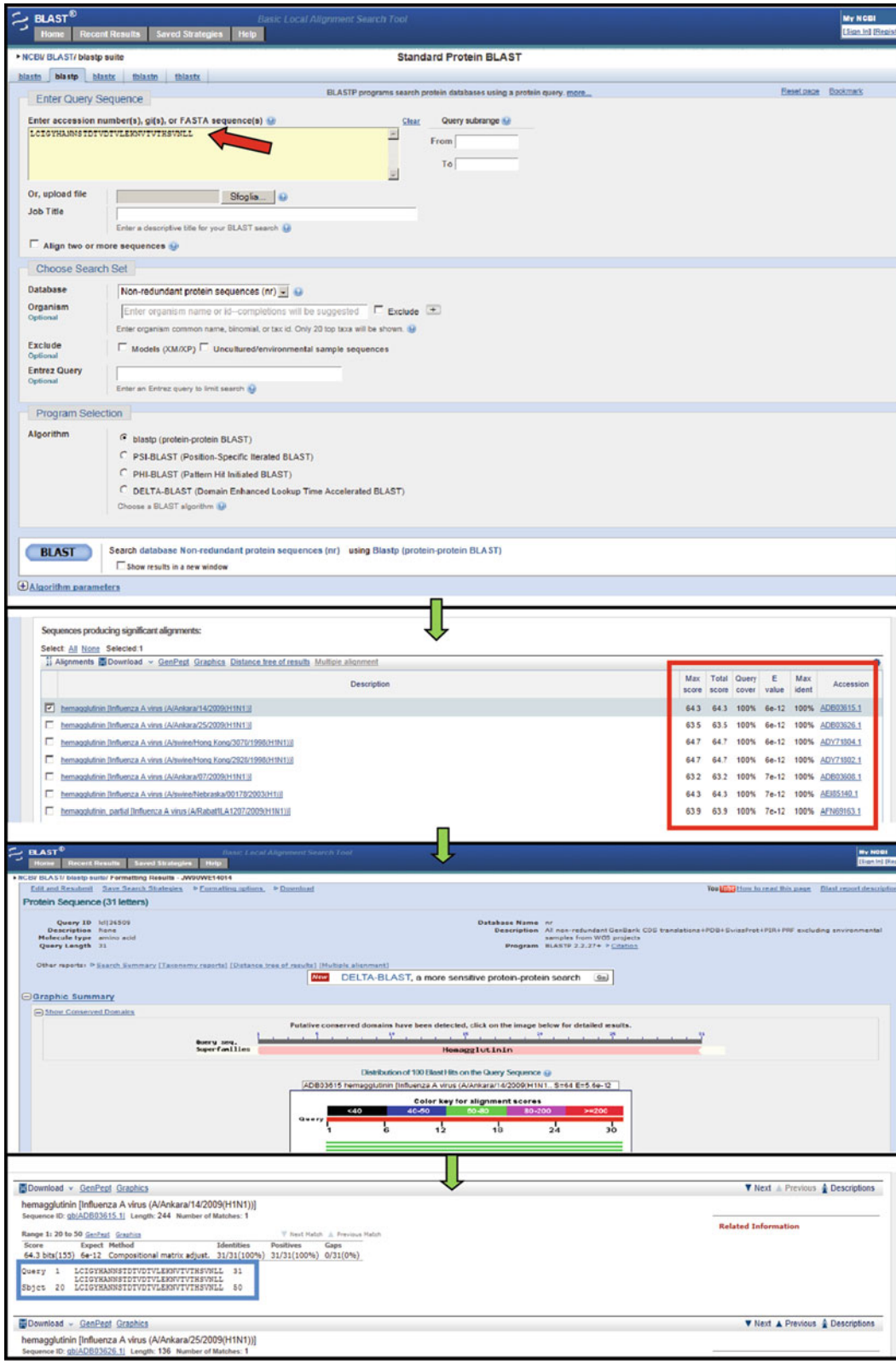

Fig. 5 Protein Blast. An example BLAST search. Once the input sequence is loaded (*red arrow*), the server will process the sequence by questing different databases. The results (*green arrows*) will highlight the database sequences showing the highest identity and higher query coverage (*red box*). Finally, after the selection of the "right" sequence, it will be possible to visualize the peptide sequence aligned with the master sequence found by Blast server (*blue box*) (Color figure online)

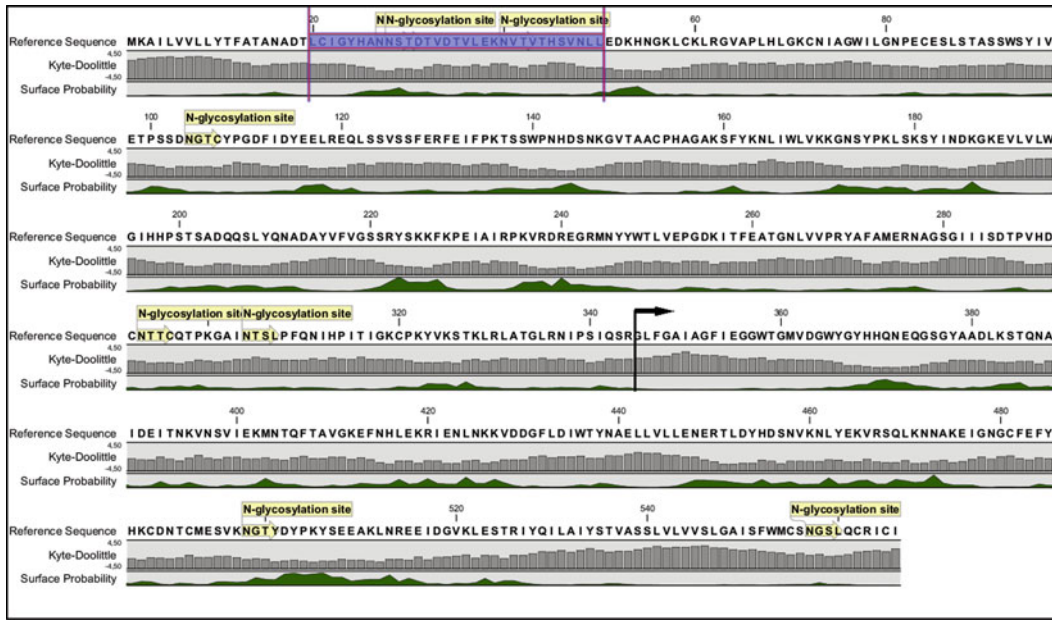

Fig. 6 As an example, the linear amino acid sequence of influenza hemagglutinin (HA)-coding region (reference sequence) has been analyzed. The output sequence deriving from the mass spectrometric approaches described above has been aligned as described in Subheading 3.4.1 and highlighted in *purple*. The HA2 domain, containing the highly hydrophobic fusion peptide, has been marked with a *black arrow*. Kyte–Doolittle analysis of the ref. sequence has been performed to evaluate the protein hydrophilic/hydrophobic regions (*boxes in grey*); moreover, the surface probability for each residues has been calculated for a first screening on the linear sequence (*graphs in green*). Finally the putative glycosylation sites have been highlighted (*yellow flags*). From this first analysis it is possible to draw possible correlations between the putative epitope and the Ab biological features (CLC Workbench) (Color figure online)

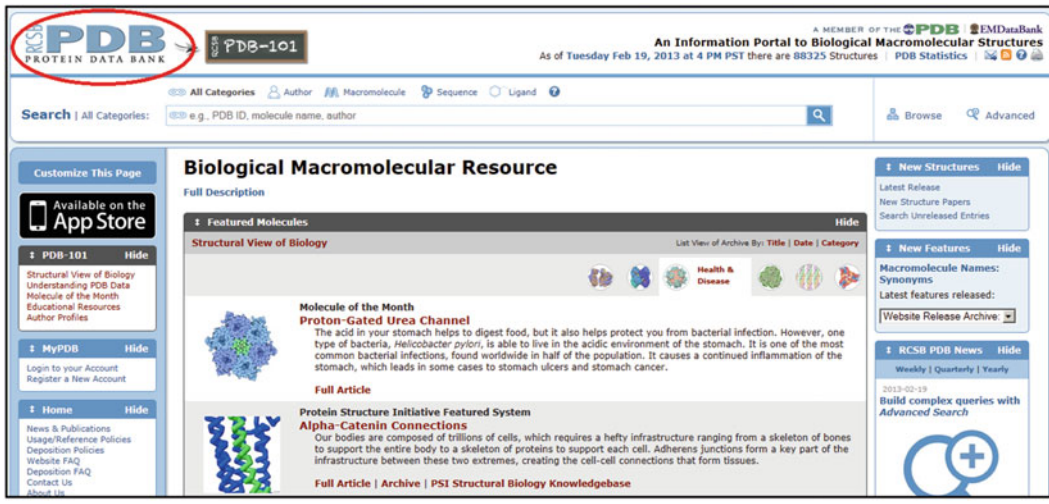

Fig. 7 Screenshot of Protein Databank. In this database it is possible to search pdb files using its pdb ID or alternatively using protein name filtering the results by categories. The file of interest supplies the references describing it and, usually, is possible to download in pdb and fasta format as well

Table 1
List of common molecular visualization and editing computer programs

Program name	Source (URLs)	References
UCSF Chimera	http://www.cgl.ucsf.edu/chimera/	Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, supported by the National Institutes of Health
Ras Mol	http://rasmol.org/	Based on RasMol 2.6 by Roger Sayle Biomolecular Structures Group, Glaxo Wellcome Research & Development Stevenage, Hertfordshire, UK
VMD	http://www.ks.uiuc.edu/Research/vmd/	Humphrey, W. et al.
Cn3D	http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml	NCBI
DeepView/SPDBV	http://www.expasy.org/spdbv/	The Swiss Institute of Bioinformatics (SIB)

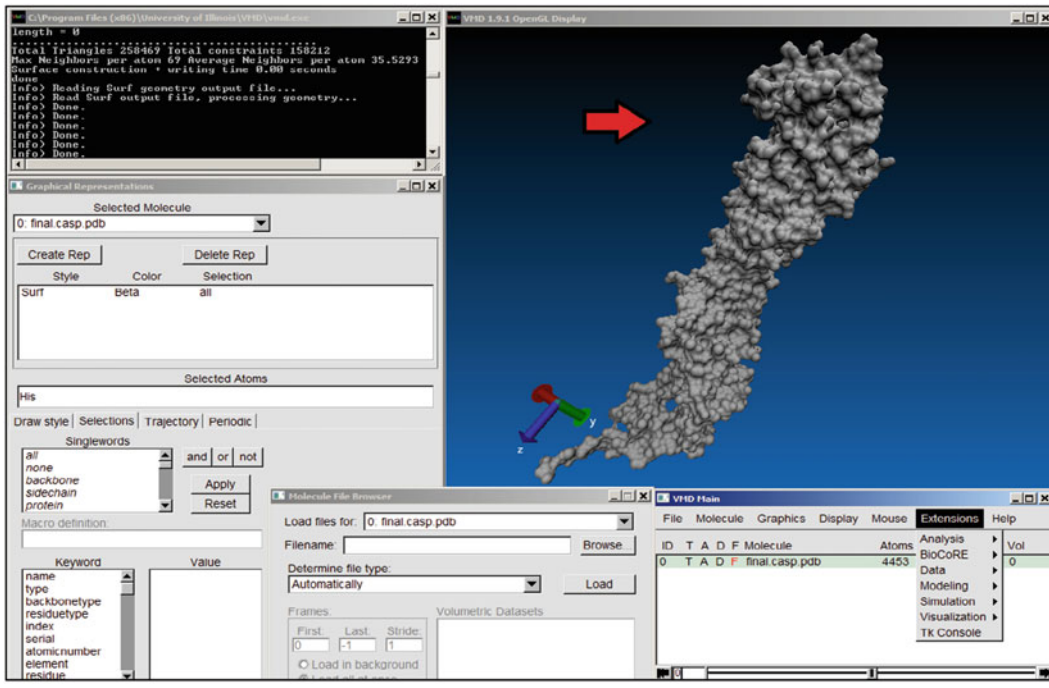

Fig. 8 The crystal structure of the Ag (*red arrow*) has been "loaded" into VMD program. In this example it is possible to appreciate the solvent-accessible surfaces (Color figure online)

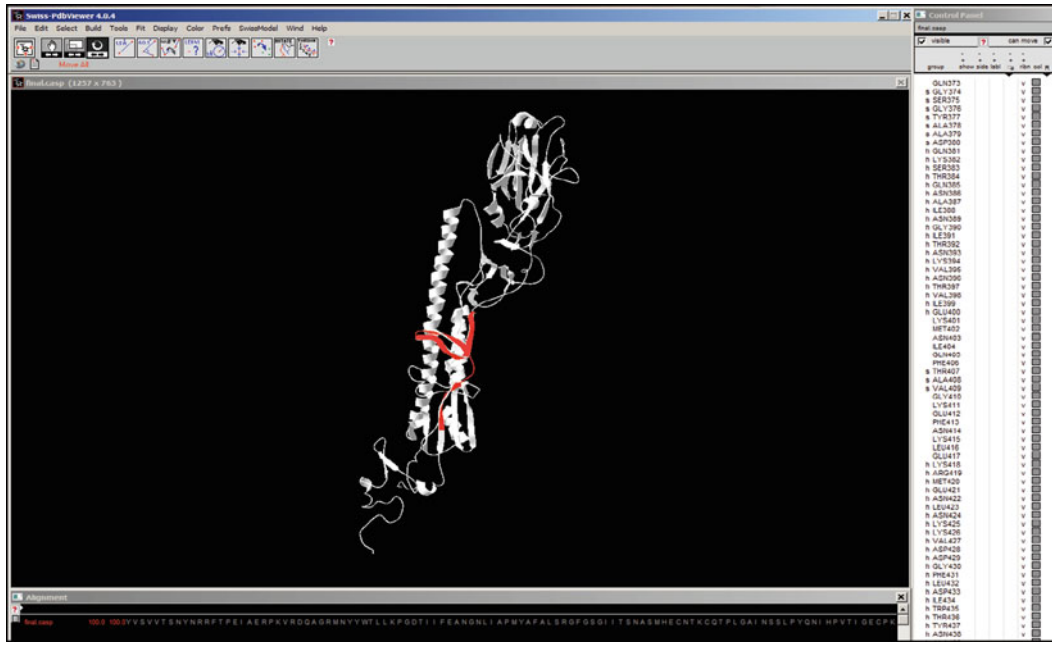

Fig. 9 The same structure (influenza hemagglutinin) edited using SPDBV. The sequence identified by the mass spectrometry experiments has been highlighted in *red*. From this picture it is possible to speculate that the epitope recognized by this Ab lies in a region not involved in the docking with the HA cellular receptor since the HA region involved in the receptor recognition lies on the "top" of this molecule (the so-called globular head). This can be easily confirmed by experimental approaches (Color figure online)

to perform the analysis of affinity-selected peptides have been developed (e.g., Mimox, http://immunet.cn/mimox; Pepitope Server, http://pepitope.tau.ac.il) [15, 16]. Generally, these tools allow for the identification of putative consensus motifs on the basis of the principal characteristics of the key consensus residues shared among the different affinity-selected peptides, by applying different algorithms. Here, we report the example of "user-friendly" server named Pepitope Server. This server is generally used to computationally predict epitopes based on selected peptide sequences extracted from a phage display library or to align a linear peptide sequence onto a three-dimensional protein structure. In brief, the first program output is the alignment of each affinity-selected phage-displayed peptide to the 3D structure of the Ag. Once few peptides have been aligned, the server adds on a clustering algorithm to find one or more patches of residues, corresponding to putative epitopes, on the surface of the 3D structure. This second output is the predicted Ab-interacting surface Ag region. Interestingly, Pepitope eliminates the non-solvent-exposed amino acid residues, assuming that the affinity-selected peptides mimic solvent-exposed amino acid residues [17]. Finally, the server allows to run three different epitope-mapping algorithms (PepSurf, Mapitope, or a combined analysis) (Fig. 10).

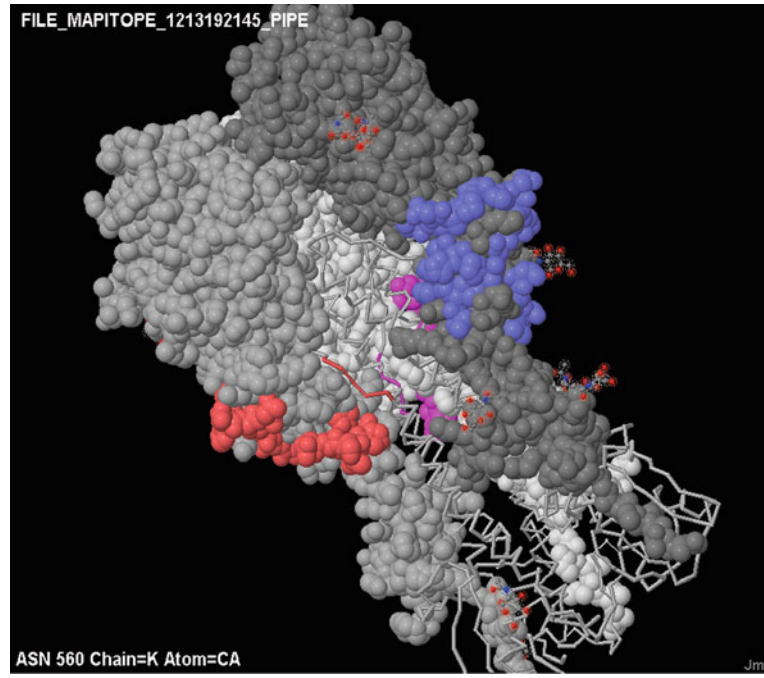

Fig. 10 Pepitope server screenshot. As described above, the Pepitope input files are Protein Data Bank files (".pdb" file extension) and the set of affinity-selected phage-displayed peptide sequences (".fasta" file format). *Red arrow* indicates the three algorithms purposed for the analysis. *Black box* indicates that it is possible to load a .pdb file or alternatively enter the Ag pdb ID. The affinity-selected peptides must be uploaded in .fasta format (*green arrow*). Several advanced settings can be also selected on the basis of the phage-displayed library used in the experimental session (Color figure online)

Once the proper algorithm is selected, the server will generate output files showing the putative epitope positions (Fig. 11).

3.4.3 *Final Notes*

As previously described [1, 8], the data generated by the examples of in silico analysis reported here must not leave aside the simultaneous generation of empirical data. Experimental results are essential to show a relationship between the epitope-mapping results and the biological features of the antibody being characterized. In addition, preliminary data generated as above can be further confirmed by experimental techniques such as alanine scanning or Pep-scan techniques.

4 Notes

1. Prepare it just before use: This step is required only for the indirect immunosorption approach.
2. For the indirect immunosorption approach, an Fc-specific antibody is required in this step in order to obtain a defined

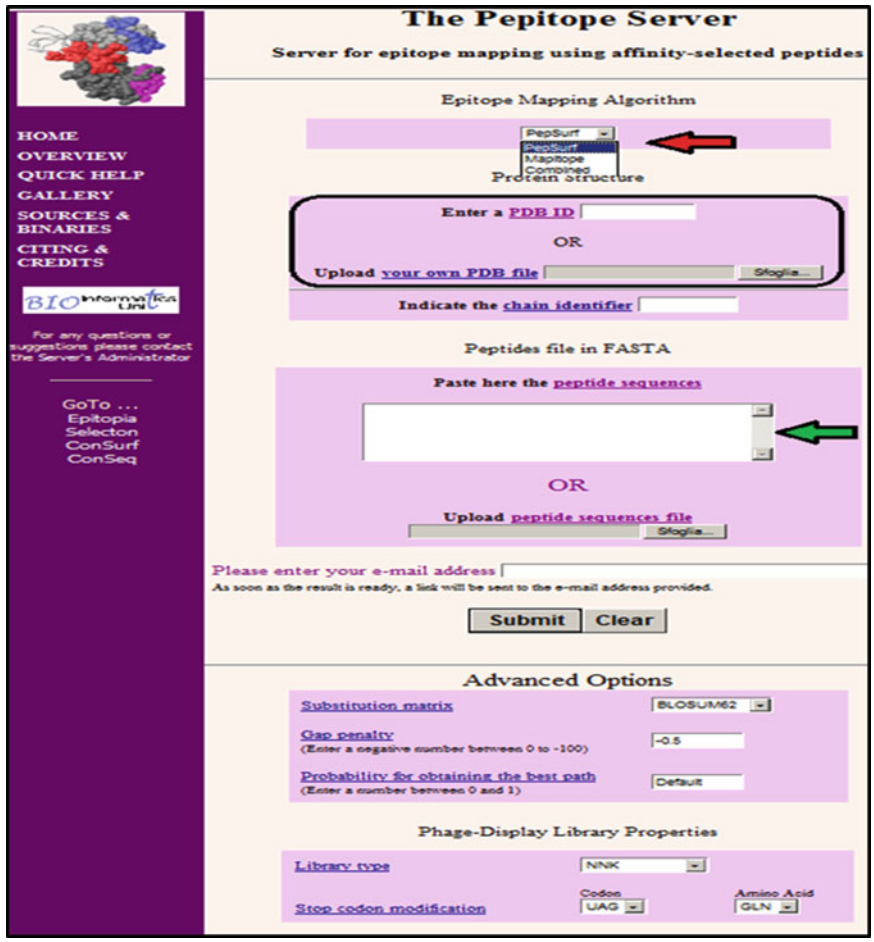

Fig. 11 Epitope (*red* and *purple*) predicted by Pepitope server's Mapitope algorithm on the influenza hemagglutinin trimeric form. Data visualized using Jmol: an open-source Java viewer for chemical structures in 3D (http://www.jmol.org/) (Color figure online)

orientation of the antibody of interest in which the paratopes are exposed to the solution.

3. This step is required only for the indirect immunosorption approach.
4. This step is required in order to block any reactive sites on the beads.
5. **Steps 11–18** are required only for the indirect immunosorption approach.
6. This step quenches the exchange reactions and elutes out the antigen from the antibody column.
7. If amplifying the eluted phage on the same day (*see* **step 12**), also inoculate 10 mL of SB medium. Incubate both cultures at 37 °C with vigorous shaking.

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