

ExSARTM

EPITOPE MAPPING USING HYDROGEN/DEUTERIUM EXCHANGE MASS SPECTROMETRY AND DOCKING

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OVERVIEW – EPITOPE MAPPING

ExSAR's proprietary platform facilitates drug development and target validation. The technology consists of an automated hydrogen/deuterium exchange ("H/D-Ex") platform coupled to proprietary software for the rapid and reproducible measure of amide hydrogen exchange rates. Typical applications involve determinations of drug binding interfaces and the detection of protein structural changes following complex formation. In this overview, application of the ExSAR H/D-Ex technology to epitope mapping including our H/D-Ex enhanced docking (HDED) methodology is highlighted.

Epitope mapping is an essential aspect of the discovery and development of diagnostic and therapeutic antibodies. Epitope mapping can streamline the selection of lead candidate molecules, particularly where epitope similarity or dissimilarity issues are involved. Intellectual property considerations of patentability, and freedom to operate consequences, can hinge upon the epitope itself. Furthermore, regulatory agencies recommend that prior to use in humans and whenever possible, the protein bearing the reactive epitope should be biochemically defined and the antigenic epitope itself determined (PTC/FDA, 94D-0259).

Commonly employed epitope mapping strategies are each associated with a particular set of limitations. For instance discontinuous epitopes, non-linear in origin and dependent upon the structural conformation of the protein, may preclude the use or complicate the interpretation of point directed mutagenesis and overlapping peptide analysis. Reported studies demonstrate that loss of binding upon mutation may not necessarily equate with the identity of the epitope. While an X-ray co-crystal of the antigen:antibody complex remains the gold standard of epitope determination, it is technically challenging, tedious and not always feasible due to the difficulty of obtaining high quality well diffracting crystals.

The purpose of this summary is to inform the reader of an epitope mapping option that unlike point directed mutagenesis, does not involve loss of binding, nor unlike crystallography, does not depend on well diffracting co-crystals. The epitope mapping method, ExSAR's H/D-Ex, employs hydrogen-deuterium exchange as a tool to identify binding interfaces. Differences in the rate of amide hydrogen exchange serve to highlight the location of an epitope. This summary provides a technical overview and in depth case studies of recent analyses. H/D – Ex is commonly referenced as a methodology of epitope determination in patents covering antibodies. One of the cases detailed was included in our client's patent application. We have also discussed recently developed HDED methodology applied on an antigen-antibody test case.

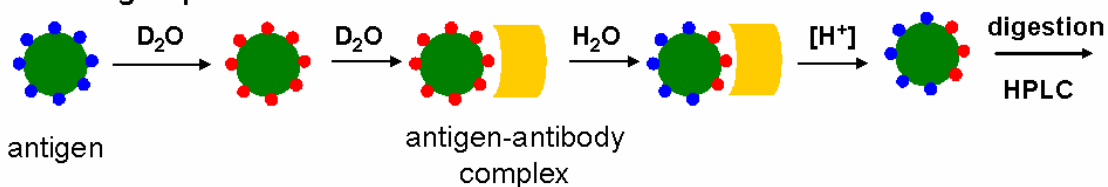
ExSAR's H/D-Ex epitope mapping technology can identify a discontinuous conformational epitope in a manner that reflects the solution state of the complex. It is a robust and efficient analytical technique that can address obstacles common to alternative techniques. Recently ExSAR has adapted this platform to yield highly accurate high resolution models of antibody-antigen complexes, effectively predicting the actual contact residues on the antigen as verified by co-crystal analysis.

I. Epitope Mapping by H/D-Ex

Upon transfer from water to a deuterium based solvent system (heavy water), a protein will experience an increase in mass as the protein's hydrogen atoms become gradually replaced with deuterons (heavier isotope of hydrogen). The likelihood of a hydrogen/deuterium exchange event is largely determined by protein structure and solvent accessibility. ExSAR's platform Hydrogen/Deuterium Exchange Mass Spectrometry (H/D-Ex) technology is used to measure exchange and as a consequence protein structure and solvent accessibility.

When a small molecule or protein binding partner binds to a protein target that target experiences experimentally observable changes in its exchange rate. Surface regions that exclude solvent upon complex formation exchange much more slowly. Solvent excluded regions are useful for deducing the location of a binding site. For instance in the case of an antigen-antibody interaction, these changes highlight the location of the epitope (see Figure 1).

<Labeling Experiment --- on-solution-off-column>



<Control Experiment --- on-column-off-column>

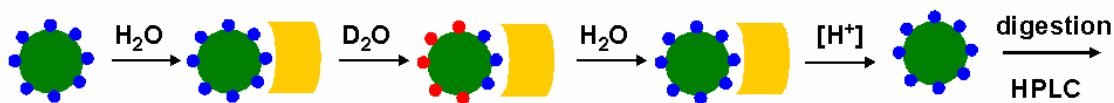


Figure 1. Concept of epitope mapping by H/D-Ex. Hydrogens are represented as blue dots, deuterons as red dots, the antigen as a green circle and the antibody as the yellow binding partner. In the labeling experiment, antigen is fully deuterated in solution and then affinity captured by column conjugated antibody. Then an H₂O based solvent is passed through the column and non-protected deuterons are washed away. In the control experiment, column captured material is deuterated and then washed with an H₂O based solvent. Very little deuterium should remain in the control sample. By comparing the H/D-Ex patterns of the labeling and control experiments, the epitope is revealed as that area of the antigen retaining deuterium.

II. Case Study-1: Epitope Mapping IgG: IL-17A complex by H/D-Ex

ExSAR was contracted by a leading pharmaceutical company to confirm a previously "determined" epitope. The epitope in question concerned a therapeutic monoclonal antibody in development against recombinant human IL-17A. IL-17A is a cytokine involved in a number of pro-inflammatory signaling pathways. Blocking IL-17A binding to its receptor, IL-17RA, suppresses inflammation, joint destruction and disease in a number of arthritis models. The strategy employed by the client was to block binding with a "neutralizing" antibody. Here the epitope was important from a development and IP perspective. The client had previously employed overlapping peptide analysis in their identification. ExSAR employed H/D-Ex to identify the epitope. The result, illustrated in Figures 2 through 4, did not agree with their earlier finding. Follow-up X-ray crystallographic studies were undertaken by the client to resolve the discrepancy. The epitope as determined by H/D-Ex was confirmed by the crystal structure of the complex. This information was included by the client in support of patent claims.

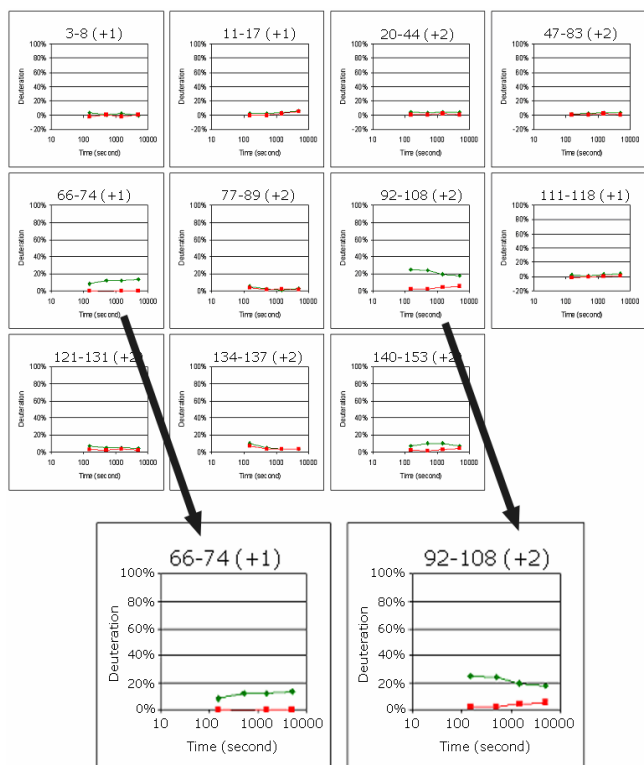


Figure 2. Deuterium content of each segment after on-off exchange experiments described in Figure 3. In on-solution-off-column experiments (green), IL-17A (antigen) was incubated in deuterated buffer for a predetermined duration, loaded onto an antibody column, and off-exchanged in the column for the same duration (e.g. 150-s-on-150-s-off, 500-s-on-500-s-off, etc.). In on-column-off-column control experiments (red), antigen was first loaded onto an antibody column, and then incubated in deuterated buffer, followed by the previously described off-exchange step. If no physico-chemical changes occur to a particular region of the antigen upon antibody binding, on-solution-off-column deuterium levels for these regions should approach zero as with the control experiment. Deuterium is retained when exchange is impeded by solvent exclusion and bonding interactions at the IgG:antigen interface. Here, segments 66-74 and 92-108 retained a significant amount of deuterium after on-solution-off-column exchange, hence leading to the conclusion that these regions likely correspond to the epitope.

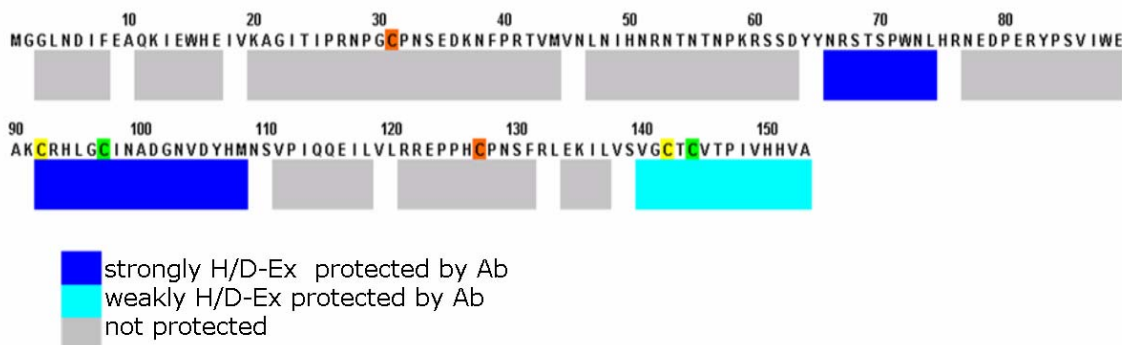


Figure 3. Anti IL-17A protects two peptides from exchange, these corresponding to amino acid sequences NRSTSPWNL and CRHLGCINADGNVDYHM and to a lesser degree protects VGCTCVPIVHHVA.

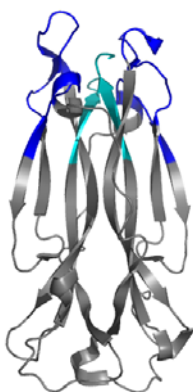


Figure 4. Strongly protected peptides are highlighted in blue and weakly protected in light-blue on the IL-17F structure (Protein Database Identifier 1JPY).

III. CASE STUDY-2: H/D-EX ENHANCED DOCKING (“HDED”) OF CytC-E8 ANTIBODY

Protein-protein docking involves computational modeling of the protein-protein complex. The structure coordinates of its component proteins can either be acquired from the X-ray crystal structures or from homology models of the proteins. Tremendous progress has been made with regard to both docking methodology and scoring function accuracy but still result in a high number of “false positives”. False positives are top scoring models, or “poses”, that deviate substantially from “reality” as determined by crystal x-ray diffraction.

As detailed in previous sections, H/D-Ex can identify a discontinuous conformational epitope in a manner that reflects the solution state of the complex. H/D-Ex has the ability to capture all the perturbations observed during protein-protein interactions including both propagated and allosteric effects. ExSAR has developed a hybridized approach that uses H/D-Ex experimental results to guide computational docking. This methodology is referred to as H/D-Ex Enhanced Docking (HDED). HDED has two important benefits. It improves the resolution of the H/D-Ex experimental technique by giving atomic details of the protein-protein interaction. Secondly it reduces or eliminates false positives inherent in conventional computational docking analyses.

The advantages of HDED are illustrated in the case study of protein-protein docking of CytC- E8 antibody. We selected this as a validation case because the high resolution co-crystal structure of the CytC-E8 antibody complex is available (PDB: 1WEJ) for confirming our results and because it is a challenging discontinuous conformational epitope. Generally, the quality of the docked poses is evaluated by Root Mean Square Deviation (RMSD) of the interface C α atoms after superposition onto the co-crystallized antigen-antibody complex. The interface is defined as all residues with at least one atom located within 10 Å of the other protein. All predictions with an interface RMSD less than or equal to 2.5 Å are generally considered “correct” poses.

Figure 5 and Table 1 compare the results obtained using HDED and computational docking. Using HDED starting with unbound forms of CytC and E8 antibody and implementing H/D-Ex results as a guide, we were able to obtain the highest ranked pose with a RMSD of 1.08 Å from the co-crystal structure of CytC-E8. As illustrated in Figure 5 and summarized in Table 1, HDED was able to correctly identify all ten CytC contact residues involved in interactions with the E8 antibody. When docking was performed in the absence of H/D-Ex data, the highest ranked pose had a RMSD of 5.36 Å from the X-ray co-crystal structure of the CytC-E8 complex. From Table 1 it can be seen that the highest ranked pose, obtained by docking in the absence of H/D-Ex, failed to identify the majority of the contact residues.

The results obtained from HDED are significant for two reasons. H/D-Ex data make it possible to filter out the false positives that typically plague computational docking. Secondly the hybridized approach of H/D-Ex enhanced docking produces a highly accurate model of the complex. This allows direct identification of contact residues. The combined methodologies yield greater resolution and accuracy than either method alone. In summary, HDED is a new and powerful tool capable of identifying an epitope with excellent accuracy and resolution.

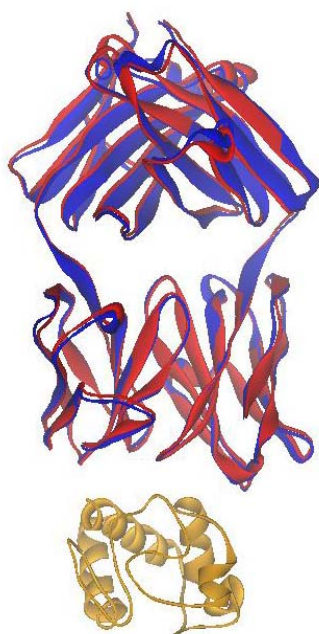
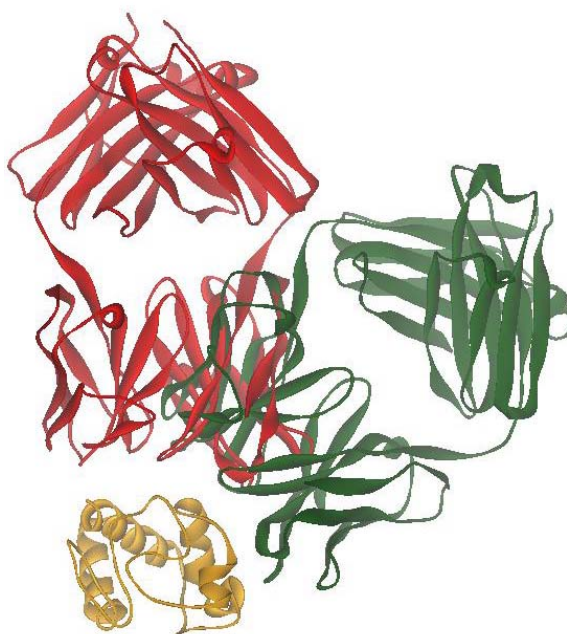
a.**b.**

Figure 5. Comparison of poses: a) CytC shown in orange, co-crystallized E8 antibody shown in red , E8 antibody from the highest ranked pose obtained using HDED shown in blue, b) CytC shown in orange, co-crystallized E8 antibody shown in red, highest ranked pose obtained without using HDED shown in green.

Table 1. Contact residues of CytC interacting with E8

CytC Contact Residue	X-RAY	HDED	Docking
Phenylalanine 36	Yes	Yes	No
Glycine 37	Yes	Yes	No
Lysine 60	Yes	Yes	Yes
Glutamate 61	Yes	Yes	Yes
Glutamate 62	Yes	Yes	Yes
Alanine 96	Yes	Yes	No
Lysine 99	Yes	Yes	No
Lysine 100	Yes	Yes	No
Asparagine 103	Yes	Yes	No
Glutamate 104	Yes	Yes	No

IV. ExSAR Competencies/Deliverables

ExSAR has offered H/D-Ex services since 2002. ExSAR has conducted over two dozen antibody based epitope mapping studies for major pharmaceutical and biopharmaceutical clients. It offers a collaborative and consultative approach, with verbal and written presentation of results. Projects are conducted under the supervision of Dr. Yoshitomo Hamuro. Its scientific leadership includes Dr. Charles Cantor, Dr. William DeGrado, and Dr. S. Walter Englander, each members of the National Academy of Science.

William F. DeGrado, Ph.D.-Chairman of the ExSAR Scientific Advisory Board

Dr. DeGrado is the George W. Raiziss Professor of Biochemistry and Biophysics at the University of Pennsylvania and he is a member of the National Academy of Sciences. His published research includes contributions to the fields of protein design, synthesis of peptidomimetics, and characterization of membrane-active peptides.

S. Walter Englander, Ph.D. – Member, ExSAR Scientific Advisory Board

Dr. Englander is the Jacob Gershon-Cohen Professor of Medical Science and Professor of Biochemistry and Biophysics at the University of Pennsylvania. He is a member of the National Academy of Sciences, Honorary Fellow of the Biophysical Society and Honorary Fellow of the American Association for the Advancement of Science. His work has focused on internal protein motions and correlations to amide hydrogen exchange rates.

Charles R. Cantor, Ph.D. - Director, ExSAR Board of Directors

Dr. Cantor is the Chief Scientific Officer and Chairman of Sequenom, and a member of the National Academy of Sciences. He was previously the chair and professor of the department of biomedical engineering and biophysics, and director of the Center for Advanced Biotechnology, at Boston University. Prior to this, Dr. Cantor held faculty positions at Columbia University. He was also director of the Human Genome Center Project of the Department of Energy at Lawrence Berkeley Laboratory. Dr. Cantor has published more than 325 peer reviewed articles and has been granted 26 U.S. patents.

Yoshitomo Hamuro, Ph.D. – Senior Director of Technology Development

Dr. Hamuro has led the development of hydrogen/deuterium exchange mass spectrometry analysis of protein dynamics, protein-ligand interactions and protein-protein interactions at ExSAR since joining in 2002. Prior to joining ExSAR, he was instrumental in the development of modern H/D-Ex technology at the University of California, San Diego, in the laboratory of Professor Virgil Woods. Dr. Hamuro conducted postdoctoral research on combinatorial chemistry and solid-phase chemistry at DuPont and later on antibacterial β -peptides at the University of Pennsylvania under Professor William DeGrado. He obtained his Ph.D. in 1996 from the University of Pittsburgh on protein structure mimetics.