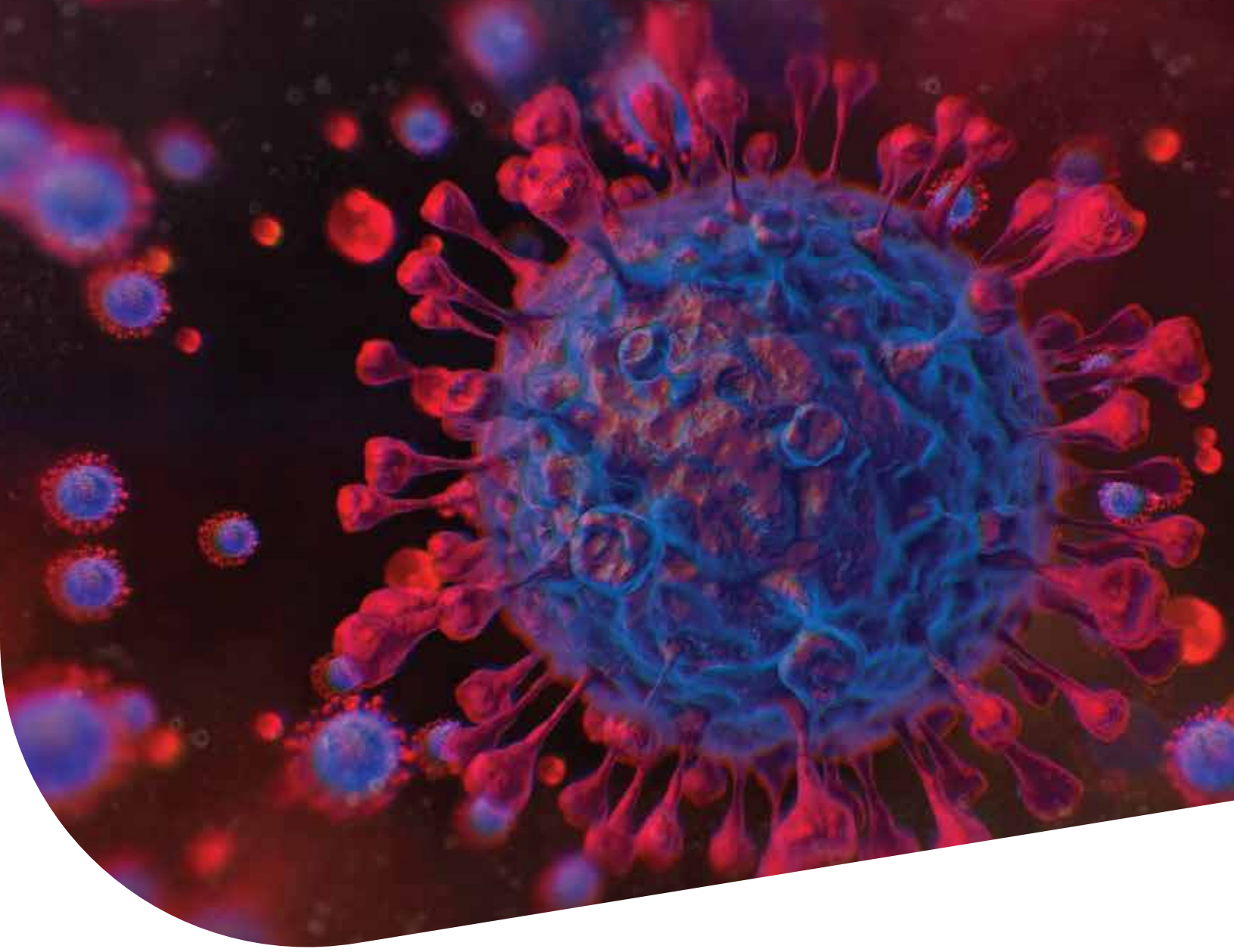


Mapping the Future

The Role of Modern Epitope Mapping Methods in Accelerating Drug Development

CovalX
Solutions for Protein Complex Analysis



Executive Summary

Over the past three decades, antibodies have emerged as a pivotal class of therapeutics in drug development. Understanding their interaction with specific epitope regions on an antigen is essential for designing novel therapies that are efficacious and safe. Additionally, epitope mapping data is typically a crucial part of the intellectual property (IP) strategy towards patenting and protecting an antibody product. Historically, X-ray crystallography has been the epitope mapping gold standard; but its complexity, cost and experimental limitations have spurred interest in alternative methods that are more accessible and can enhance research outcomes.

Recent research by scientists at Merck indicates that cross-linking provides more accurate epitope identification compared to crystallography, with greater accessibility. However, cross-linking is not a one-size-fits-all solution. Various other epitope mapping methods offer distinct benefits related to accessibility, speed, and result detail. Moreover, different methods are more suitable for specific protein interactions, such as size limitations or if the structure is unknown.

To support bioanalytical scientists in enhancing their protein interaction analysis capabilities, this whitepaper aims to evaluate the effectiveness of chemical cross-linking against other advanced epitope mapping methods commonly employed in drug development. Our findings provide actionable insights to enhance protein interaction analysis and accelerate drug development timelines.

The Power of Epitope Mapping

Accelerating Market Access, Improving Patient Outcomes & Staying Competitive

Accurate insights into protein:protein interactions (PPi) play a pivotal role in the competitive landscape of proteomics, offering crucial understanding of therapeutic antibody mechanisms and streamlining drug development processes. Robust data on these interactions not only enhance research scope but also ensure drug safety, efficacy, and quality; thereby expediting regulatory approval and improving patient outcomes.

Precise protein characterization methods are indispensable for obtaining regulatory approval for new protein-based therapeutics. Enhanced accuracy also accelerates adoption rates by fostering trust and bolstering the reputation of novel products among healthcare professionals, patients, and stakeholders. This trust is essential for facilitating market penetration and ensuring long-term success in a competitive industry landscape.

The versatility of modern characterization methods provides the opportunity to produce market firsts with sustainable optimization potential, allowing drug developers to remain at the forefront of the market indefinitely. This potential for refinement ensures ongoing innovation, crucial for scientific progress and better patient outcomes over time. Ultimately, the choice of epitope mapping method is one of the most important decisions a company can make, directly impacting time to market, regulatory compliance, patient outcomes, and the future standing of the company.



Regulatory Considerations

When choosing a method to elucidate immunochemical properties of a protein in development, there is a baseline of accuracy and precision necessary to comply with characterization requirements laid out by the FDA and other regulatory agencies. ICH-Q6B guidelines stipulate that "binding assays of the antibody to purified antigens and defined regions of antigens should be performed, as feasible, to determine affinity, avidity and immunoreactivity (including cross-reactivity)." While all of the methods we have described here should be able to meet regulatory requirements, they vary specifically in three domains: time, accuracy and cost.

Patent Application

As the $100B therapeutic monoclonal antibody (mAb) market continues to grow, developers of therapeutic mAbs increasingly face the need to strengthen patent protection of their products and enforce their patents in courts. Legal battles, such as those involving Sanofi's patents against rival monoclonal antibodies (mAbs) that block PCSK9 activity, have highlighted the central role of epitope mapping in establishing broad mAb patent rights (Deng, Storz & Doranz, 2017). These cases illustrate that for a patent claim to be robust, it must not only describe a sufficiently large number of mAbs sharing an epitope but also delineate each epitope.

Epitope mapping information is vital for drafting strong and defensible patent applications. The identification of specific amino acids within an epitope, as enabled through high-resolution epitope mapping, supports precise claims. Such detailed claims are crucial for safeguarding intellectual property in the competitive field of antibody therapeutics.



Choosing the Best Technique for Your Needs

> ***"The target molecule bearing the relevant epitope should be biochemically defined and the epitope itself defined, when feasible."***
> *- FDA, Guidance for Industry ICH-Q6B*

Innovative Methods for Determining Protein Structure

While X-ray crystallography has historically been viewed as the gold standard for epitope mapping, the process of obtaining a crystal structure for an antibody-antigen complex is time-consuming, boasts a moderate success rate, and demands significant resources and expertise. As a result, alternative approaches have been developed. Here, we offer a comprehensive guide to compare and contrast the benefits, applications, and limitations of three modern epitope mapping methodologies: Cross-Linking Mass Spectrometry (XL-MS), Hydrogen-Deuterium eXchange Mass Spectrometry (HDX-MS), and alanine scanning.



Cross-Linking Mass Spectrometry (XL-MS)

"Our results suggest chemical cross-linking most accurately identifies the epitope as defined by crystallography." *- Merck, Epitope Mapping of Monoclonal Antibodies a Comprehensive Comparison of Different Technologies.*

Key Considerations

1. XL-MS is considered the most accurate method compared to crystallography.
2. Coss-linkers are able to target several different amino acid residue types.
3. Both epitope and paratope data are detected simultaneously in one experiment.
4. No need for crystallization, mutation or peptide generation means defined timelines and material requirements.
5. Lowest material and purity requirements for any epitope mapping technique.
6. Trusted MS/MS fragmentation data from high resolution mass spectrometry provides increasing confidence in results.

Technique Overview

Cross-Linking Mass Spectrometry (XL-MS) is an experimental structural analysis workflow utilized to identify the interaction sites from constituents of macromolecular complexes. XL-MS begins by stabilizing a non-covalent interaction between two proteins using chemical cross-linkers. The cross-linkers covalently bind specific known residues on each protein partner providing a defined interactions on or near the complexes interactions site.

The use of a mixture of deuterated and undeuterated crosslinkers provides two distinct (doublet) peaks from these two different crosslinker masses which can be distinctly identified. Both the deuterated and undeuterated cross-linkers are bound stably to the protein allowing clear detection even throughout the MS/MS fragmentation of specifically which residues the cross-linkers are binding on each peptide. Special software is then utilized to track and detect these doublet peaks on the peptides detected after MS/MS.

Benefits

The XL-MS workflow can serve multiple purposes, including identifying sites of protein dimerization, comparing protein conformations in different conditions, and obtaining distance constraints for modeling protein structure and conformation of protein complexes. One of the most common and valuable uses of the crosslinking technology is the characterization of antibody:antigen interactions to clearly define their epitope:paratope interactions.

As the peptide interactions can be very accurately verified using high-resolution MS/MS techniques, there is also a high degree of trust in the XL-MS data. Additionally, because XL-MS is conducted directly on the desired proteins in solutions without the need of generating alternative mutations, crystals or peptide mimics, the measurements are directly from the desired sample materials.

Applications

XL-MS is suited to a few specific applications. Crosslinking experiments do require the sequence information of both interacting partners to be known, and disulfide bonds or glycans can affect peptide detection. Additionally, XL-MS can not be conducted in the presence of amine-containing buffers, so it may be required to rebuffer materials prior to

analysis. Finally, while XL-MS provides data on the exact residue where the crosslinker is binding,this crosslinking residue is not necessarily the exact epitope site which can be within 2-3 residues. Additional validation with techniques like HDX-MS or alanine scanning may be helpful for higher resolution validation.



Hydrogen-Deuterium eXchange Mass Spectrometry (HDX-MS)

Key Considerations

1. Can be used to study protein-protein or protein-ligand interactions.
2. Provides insights into protein conformation and flexibility, as well as binding sites.
3. Replicates using automated analysis for improved consistency and throughput.
4. Established technique used in hundreds of labs worldwide for decades.
5. High-resolution mass spectrometry, robotics and dedicated software provides trusted confidence in results.

Technique Overview



Hydrogen-Deuterium eXchange Mass Spectrometry (HDX-MS) leverages hydrogen-to-deuterium exchange directly in solution to assess alterations in protein flexibility, conformation and interactions. This technique determines uptake of deuterium from backbone amine groups by measuring the mass differences of deuterium versus hydrogen across peptides using mass spectrometry.

The general workflow entails labeling the target protein (i.e. antigen) or protein-ligand complexes with deuterium from deuterated water (D_2O), followed by denaturation and quenching at low temperatures under acidic conditions.

Subsequently, proteolysis is carried out using pepsin digestion, and the resulting peptides are separated and analyzed using high-resolution MS/MS mass spectrometry. Automated data analysis ensures consistency, allows for replicates and maximizes instrument throughput for expedited results.

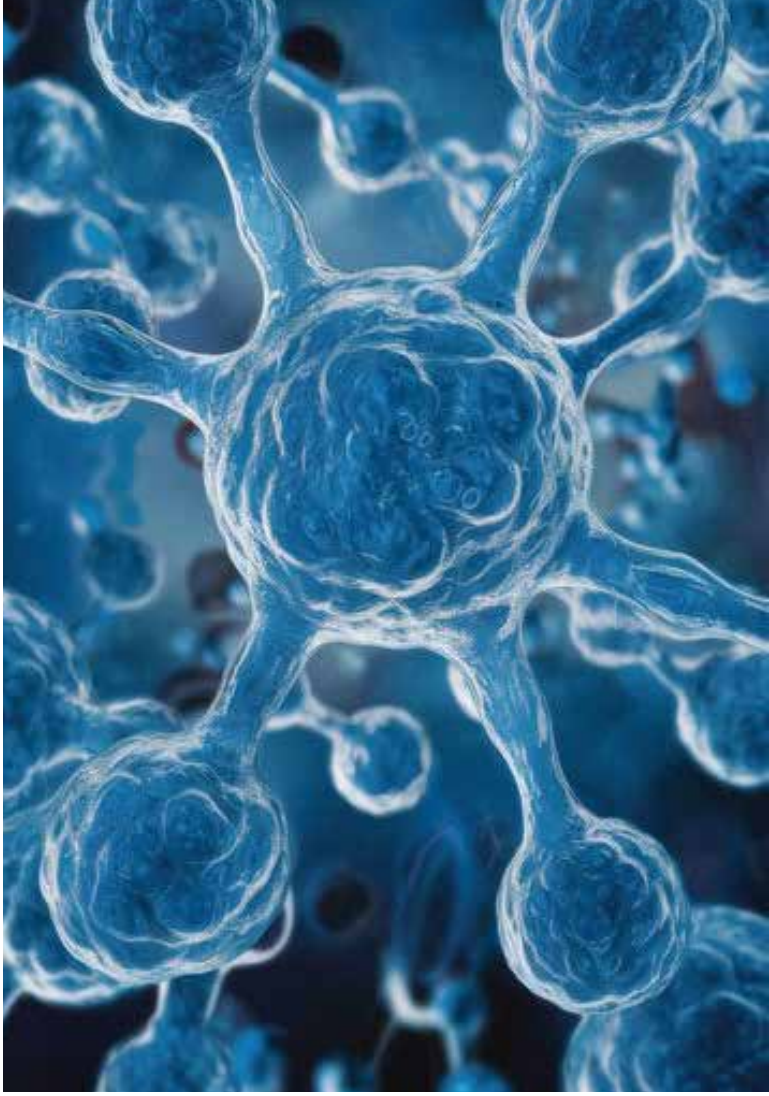
Benefits & Applications

HDX-MS offers a plethora of applications in protein analysis, including identifying protein folding, detecting conformational changes post-events, locating binding sites, mapping protein aggregation sites, assessing the effects of mutations, and characterizing sites of protein-drug interactions. One of the most valuable uses of the HDX-MS technology is the characterization of antibody:antigen interactions to clearly define their epitope interaction on monoclonal antibodies (mABs).

Over several decades, HDX-MS has become an established and trusted technique for most of the major pharmaceutical companies and research institutes. By comparing results with unlabeled peptides, detailed information about ligand binding and exchange sites can be elucidated, providing valuable insights into protein dynamics and interactions.

Limitations

Flexible or unstructured proteins, which can exhibit more dynamic structures which may pose challenges for HDX-MS analysis in those specific regions. Increased flexibility can lead to higher rates of hydrogen back exchange, resulting in broader and less defined HDX-MS patterns. Consequently, interpreting data from some protein regions may be more challenging and therefore each protein must be evaluated individually. Additionally, disulfide bonds and glycans may make peptide detection difficult in some regions of a protein. Finally, very large proteins (i.e. greater than 100kDa) may be difficult to generate and detect peptides over the entire protein sequence due to the limited digestion conditions required during HDX-MS analysis.



Alanine Scanning

Key Considerations

1. Protein engineering methods involve the substitution of alanine residues.
2. Alanine substitution may increase the risk of false positives.
3. It supports studies focused on single protein-protein interactions.
4. Accuracy and throughput can be limited due to dependency on traditional techniques such as ELISA.
5. Mutant versions must be generated, reducing overall protein material requirements.

Technique Overview

Alanine scanning is a protein engineering method employed to pinpoint specific residues within an antigen crucial for binding to an interacting partner, such as an antibody. This technique involves systematically substituting individual amino acid residues with alanine, a small, nonpolar amino acid. Subsequently, variants with alanine substitutions are assayed to assess any changes in binding affinity compared to the wild-type protein. A decrease or loss in binding affinity indicates that the substituted amino acid likely contributes to the interaction interface and the epitope recognized by the antibody.

Benefits & Applications

Alanine scanning can offer the advantage of providing a detailed array for each specific target protein. These arrays can then be used rapidly for screening larger numbers of interacting partners once generated. While traditionally reliant on techniques like enzyme-linked immunosorbent assay (ELISA) for binding analysis, which are familiar but less precise, alanine scanning can provide more detailed insights into the interaction interface. It allows researchers to identify specific amino acids critical for binding, aiding in the design of targeted therapeutics or diagnostic tools.

Limitations

Despite its utility, alanine scanning has several limitations that must be considered. Firstly, substituting amino acids with alanine may not fully mimic the native chemical properties of the replaced residues, potentially leading to false-positive results. Moreover, alanine scanning may not capture all critical amino acids involved in binding interactions, particularly in cases where cooperative effects or interactions involving multiple amino acids occur making detection of discontinuous binding sites unreliable. Finally, compared to more advanced techniques such as MS, alanine scanning lacks the reliability and validation necessary for comprehensive epitope mapping studies.



Overview of Epitope Mapping Methods

Fastest	Most Accurate	Most Cost-Effective (for known proteins only)
HDX-MS or XL-MS	Cross -Linking MS (XL-MS)	Alanine Scanning

Discussion

Ultimately, selecting the optimal method for epitope mapping revolves around creating top-tier technologies to address unmet needs in a rapidly evolving market. Companies must determine which method offers the most efficient route to market, achieve regulatory and intellectual property requirements, and accelerate adoption. The ideal method will align with your budget, timelines, and company objectives, while also delivering reliable results.

Navigating the landscape of epitope mapping methods poses challenges, further compounded by the scarcity of expertise in this specialized domain which have established operations within the United States (US). Although partnering directly with overseas CROs may initially appear expedient, selecting an international partner introduces logistical hurdles, data security concerns and supply chain risks that could potentially compromise project timelines and objectives.



An Established Partner With Epitope Mapping Expertise

As an established contract research organization with US and European locations and almost 20 years of experience focused on protein interaction analysis, Covalx has emerged as a visionary leader in epitope mapping offering several specialized services and technologies. Our comprehensive services, supported by extensive expertise and a commitment to innovation, keep our partners at the forefront of the evolving protein therapeutics market.

With locations on the East Coast (Boston) and in Europe (France and Switzerland), we ensure seamless collaboration and reliable services nationwide and worldwide, eliminating project delays due to logistical challenges.

Our unique expertise in XL-MS and HDX-MS is highly sought after by pioneering biotech companies seeking a boutique partner capable of providing personalized attention and fostering enduring customer relationships.

We hope you have found this resource useful. If you have any questions, please feel free to reach out to a member of our team to discuss unique considerations for your project.



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