Designing A Better Assay- HLA-DR Binding Assays as a Tool for Immunogenicity Screening: Developing the Best Practices for Determining Immunogenicity

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Introduction

Purpose

- As generic drugs and protein therapeutics become more readily available, anti-drug immunogenicity becomes a greater concern.
- HLA-type has been correlated with immunogenicity and several in silico and in vitro methods exist for screening protein therapeutics for the presence of HLA-binding peptides. (Cousens et al 2006)
- While multiple in vitro methods for assessing HLA binding are in use, **not all are created equally**.
- Knowing which methods provide the most accurate and reliable results is important for assessing the assay's reliability.

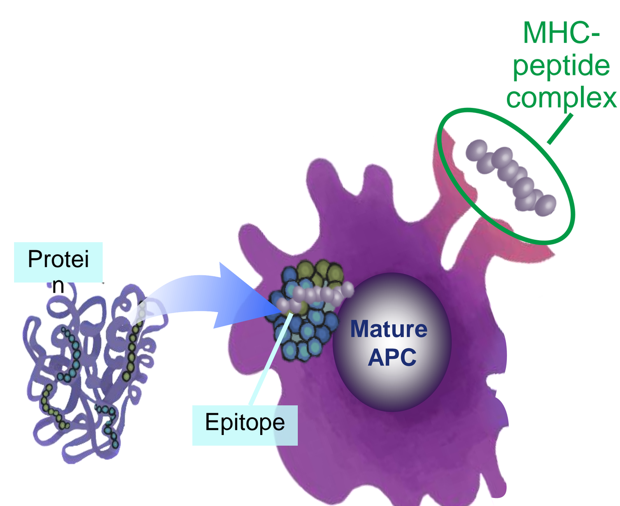
Objectives

1) To compare and contrast several methods in use to measure HLA –binding in vitro
   a. Single concentration vs dilution curve
   b. Overlapping peptide libraries
      i. The importance of properly centered peptides

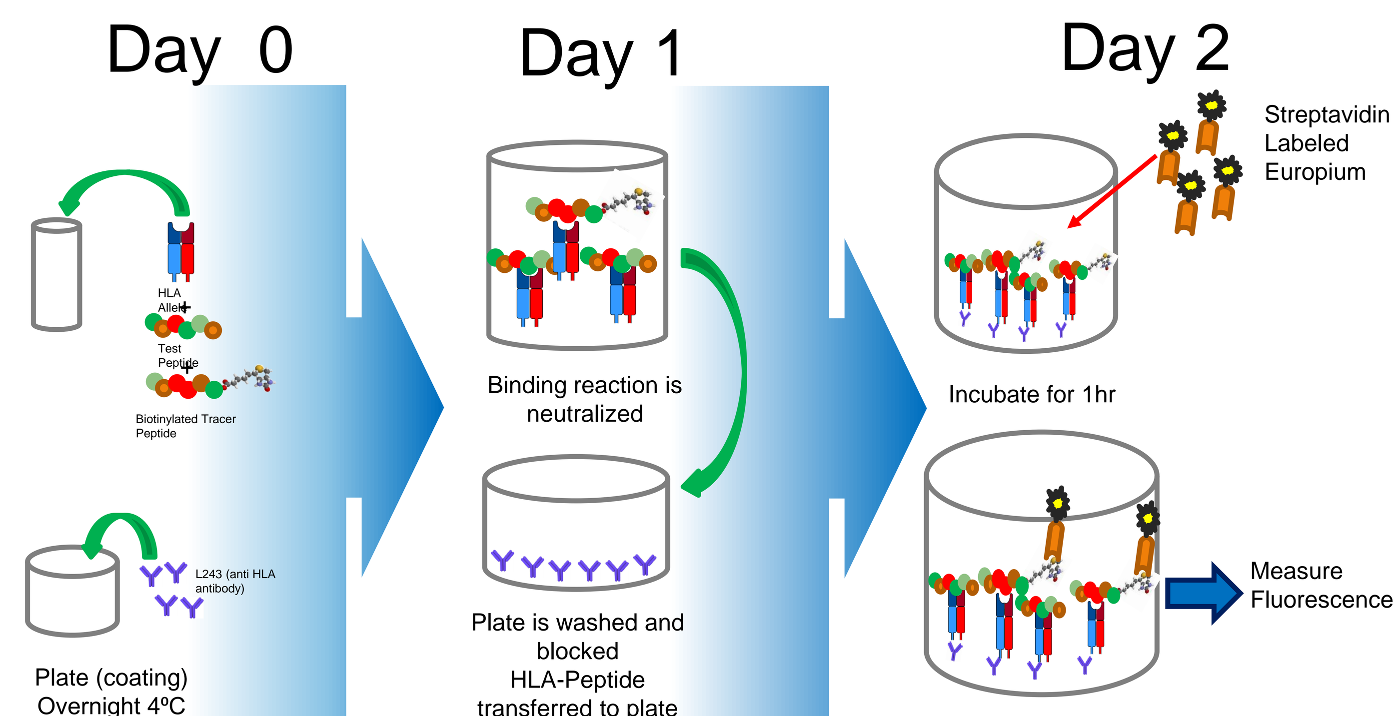
Methods

Immunogenicity assessment:

- **EpiMatrix** takes a protein sequence and parses it into overlapping 9-mer frames. Peptides are analyzed for their ability to bind 8 MHC-II supertype alleles.



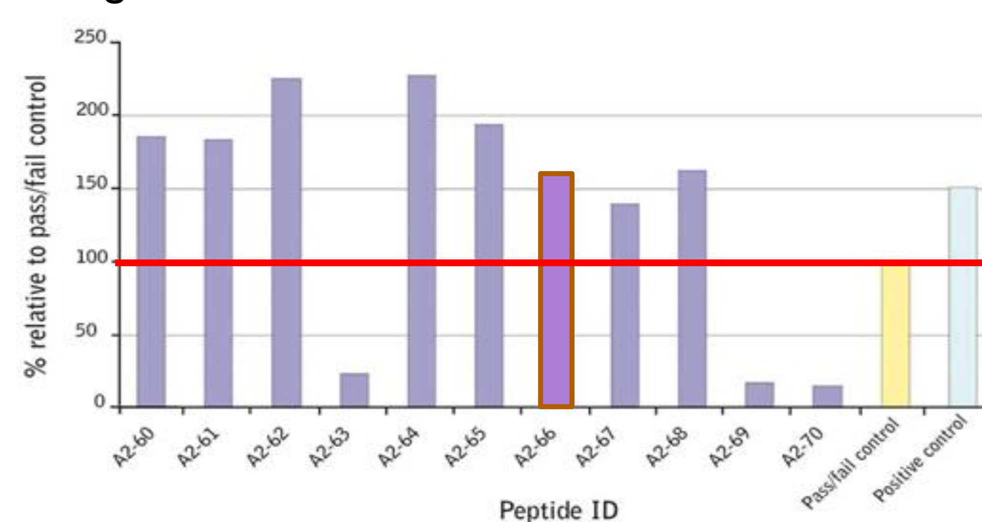
- **HLA Binding Assay**: Peptides that are predicted to bind MHC are synthesized and assayed over a range of 7 concentrations in our HLA-binding assay. In brief, peptides are incubated overnight with soluble HLA and a biotin labeled competitor of moderate affinity. On day 2, the reaction is halted and the mixture is transferred to a plate coated with a pan anti-HLA antibody. On day 3, plates are developed by the addition of streptavidin-Europium and fluorescence is measured.



Assay Sensitivity: Single Concentration vs Dose Range

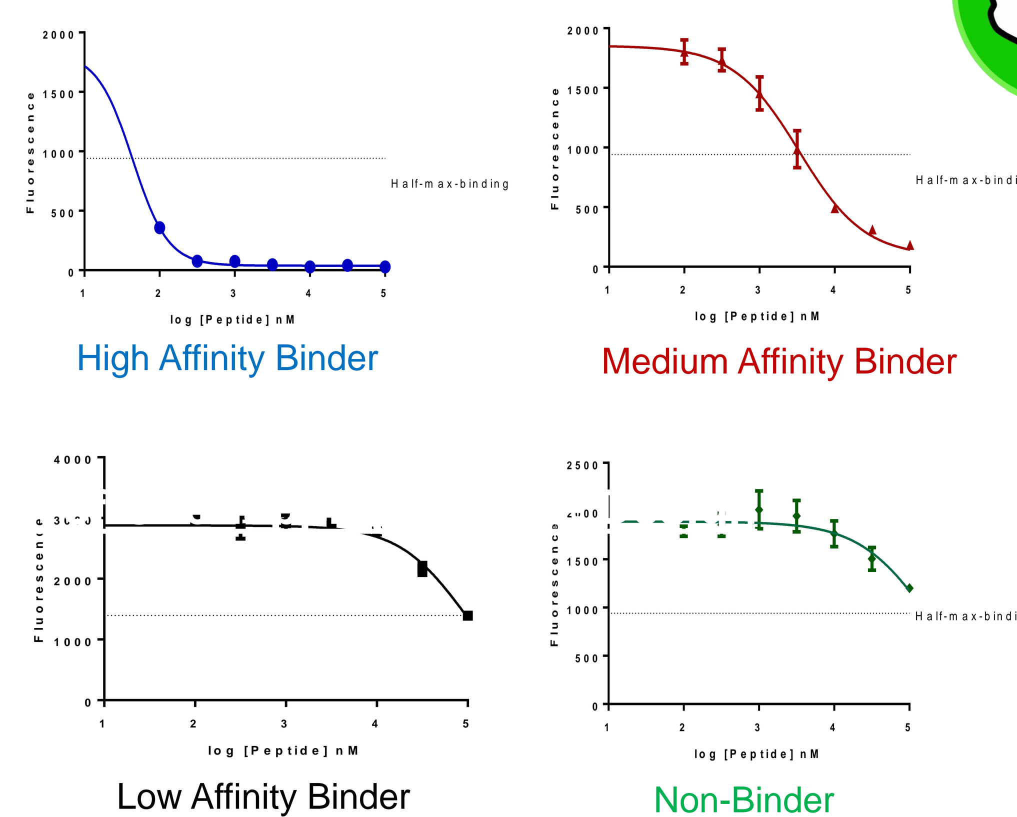
Poor Design

Figure 1a



Best Design

Figure 1b



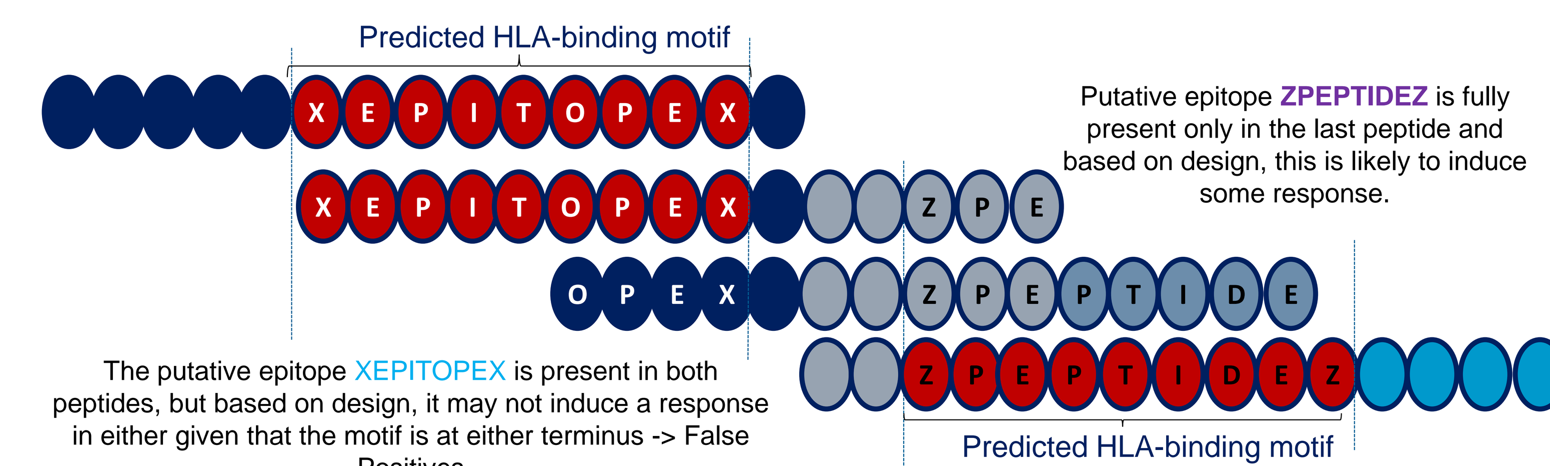
Non-binder (No dose-dependent inhibition)
Negligible Affinity (100,000 nM < IC_{50})
Low Affinity (10,000 nM < IC_{50} < 100,000 nM)
Moderate Affinity (1,000 nM < IC_{50} < 10,000 nM)
High Affinity (100 nM < IC_{50} < 1,000 nM)
Very High Affinity (IC_{50} < 100nM)

IC50 values and peptide binding affinity

Figure 1. (a) Assaying peptides at a single concentration requires measuring binding relative to a pass/fail control. Peptides that don't meet this cutoff value (red line) would be considered "non-binding" and excluded from further screening why they could contribute to the immunogenicity of the drug. (b) Measuring peptide binding over a range of concentrations provides information about the peptide-HLA affinity and allows us to classify peptides as either strong, moderate, weak or non-binders.

The Drawback of Overlapping Peptide Design

Poor Design



Overlapping (OL) peptide libraries contain binding motifs that are not properly centered. Peptides that bind to HLA Class II molecules are stabilized by flanking residues. When considering synthetic peptides for in vitro testing and validation, poorly centered HLA-binding motifs may result in absence of binding or T cell response despite the presence of an HLA binding motif. Synthesizing OL peptide libraries is also costly and time consuming.

An example of overlapping peptide data is shown below. In this example, each peptide overlaps its neighbor by 14 amino acids therefore peptides 7 and 9 should contain the same nine-mer found in peptide 8 that bind to HLA.

Best Design

ORIGINAL — EpiMatrix Cluster Detail Report, RH36-50 Cluster: 36

OPTIMIZED — EpiMatrix Cluster Detail Report, RH36-50MOD Cluster: 33

ORIGINAL — EpiMatrix Cluster Detail Report, 07_IL46-60 Cluster: 46

OPTIMIZED — EpiMatrix Cluster Detail Report, 08_IL46-60MOD Cluster: 43

Strong binding motif (EpiBar) located at flanks

IC50 values obtained qualify as binders according to EpiVax criteria

Optimized Peptide has a centered binding motif

With optimized version, we find binders with stronger affinities

Four more binders observed with repeat assay

With optimized version, we find more binders with stronger affinities

Publication Results — HLA-DR alleles: DR1, DR4, DR7, DR11, DR15; Peptides: RH36-50, IH41-55, IH46-60, IL31-45, IH51-65, IL46-60, RL56-70, RL46-60 (Three binders found (unspecified))

Positive Predictive Value (excluding RL46-60): 22%

Optimized Peptides Results (EpiVax) — HLA-DR alleles: DR1, DR4, DR7, DR11, DR15; Peptides: RH36-50, IH41-55, IH46-60, IL31-45, IH51-65, IL46-60, RL56-70, RL46-60

Positive Predictive Value (including RL46-60): 83%

True Positive or True Negative

False Negative (top 10% EpiMatrix hit)

False Positive/False Negative

Note: Only a subset of the data is shown here and we are only reporting data for optimized peptides

In a study by Hamze et al ("original") we observed a poor correlation between in silico predictions and HLA-binding. When peptides were properly centered ("optimized"), we see improved correlation between the in silico and in vitro results. Optimizing peptide design leads to stronger correlation and higher binding affinities

Conclusions

- Assay design is critical for correlating in silico and in vitro HLA binding data.
- To draw the most accurate conclusions on immunogenicity towards biologics, the industry must adopt a consistent and standard set of best practices for peptide and assay design.

References

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Steere AC, Klitz W, Drouin EE, et al. Antibiotic-refractory Lyme arthritis is associated with HLA-DR molecules that bind a Borrelia burgdorferi peptide . *J Exp Med* 2006; 203: 961–971.

Hamze M, Meunier S, Karle A, et al. Characterization of CD4 T Cell Epitopes of Infliximab and Rituximab Identified from Healthy Donors. Frontiers in Immunology. 2017;8:500.