High Resolution Epitope Mapping and Antibody Cross-Reactivity Analysis

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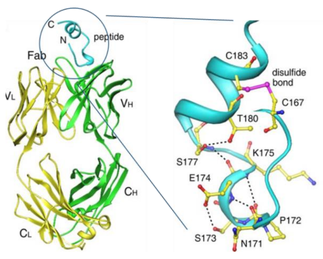
Abstract

Antibodies are among the most important life science tools for therapeutics, basic research and diagnostic tests. However, mono- and polyclonal antibodies are often poorly characterized in terms of specificity and cross-reactivity, needing further validation and cross-reactivity analysis. High density peptide microarrays are ideally suited for antibody characterization, enabling the analysis of epitope-antibody interactions with unmet speed and precision. Based on approaches for linear and conformational epitope mappings as well as high resolution epitope substitution scans, we developed a comprehensive toolbox for the in-depth analysis of epitopes and antibody cross-reactions for all kinds of antibodies and isotypes in the most flexible, comprehensive and economic manner. Moreover, we designed a Human Epitome Microarray comprising all linear human B-cell epitopes of the Immune Epitope Database (23,163 linear peptides), complemented by 4,661 epitopes of the most common vaccines. In a three-step approach based on a single assay, the Human Epitome Microarray enables a very detailed cross-reactivity analysis of antibodies including the identification of antibody-specific consensus motifs and database blasting to identify possibly cross-reacting human antigens.

Characterization of Rituximab by Conformational Epitope Mapping and Epitope Substitution Scan

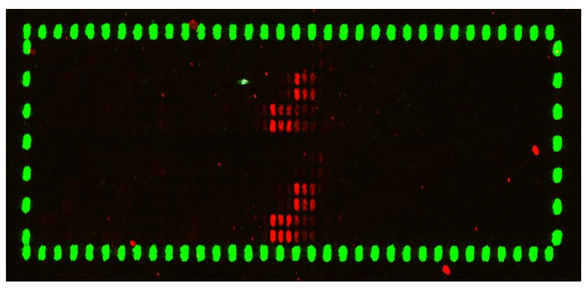


- Rituximab (Rituxan®), a chimeric monoclonal anti-CD20 antibody, is known to interact with the 15 amino acid loop of the CD20 antigen (Du et al., 2007): NIYNCEPANPSEKNSPSTQYCYSIQ (Figure 1).
- High resolution epitope mapping with peptide microarrays carrying linear and cyclic constrained overlapping peptides (7 aa, 10 aa and 13aa) of the CD20 antigen.

Figure 1: Structure of the Rituximab Fab-CD20 epitope-peptide complex (Du et al., 2007).

Results Epitope Mapping

- Linear CD20 peptides did not show any response with Rituximab (data not shown), however constrained cyclic CD20 peptides show a very clear and strong response.
- High resolution data identifying **EPANPSEK as Rituximab epitope**. Clear epitope peaks for constrained cyclic peptides with excellent signal to noise ratio (Figure 2).



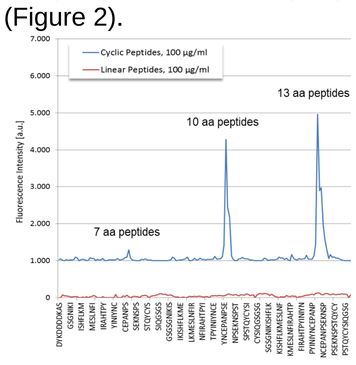
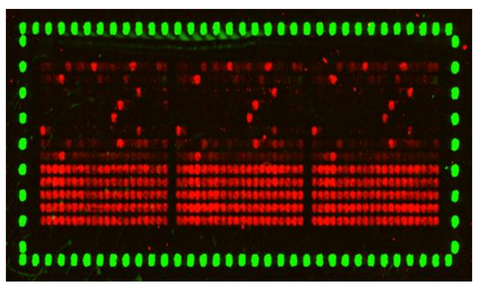


Figure 2: Peptide Microarray of cyclic CD20 peptides. Array was incubated with Rituximab followed by staining with anti-human IgG (red) and anti-Flag (green) antibodies. First double-row in each half corresponds to the 7 amino acid peptides, the second and the third double-row to the 10 and 13 amino acid peptides. Intensity plots of the Rituximab assays with linear and constrained cyclic CD20 peptides (right).

Results Epitope Substitution Scan

- In-depth epitope analysis of the constrained cyclic CD20 peptide NIYNSEPANPSEK led to a clear and well-defined substitution pattern.
- Array and amino acid plot reflect the conserved core motif EPANPSEK as well as a variable amino acid stretch NIYNC at the N-terminus (Figure 3).



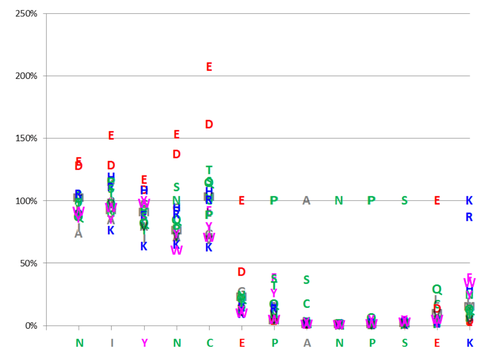


Figure 3: Epitope Substitution Scan of wild type Rituximab peptide NIYNCEPANPSEK (left). Each amino acid position was gradually exchanged by the 20 L-amino acids. The same substitution pattern is visualized by an amino acid plot (right).

Antibody Cross-Reactivity Profiling of a Human Monoclonal Antibody with the PEPperCHIP® Human Epitome Microarray

- Cross-reactivity analysis of a human monoclonal anti-c-Myc antibody with the PEPperCHIP® Human Epitome Microarray in a three-step approach (Figure 4):
  - The Human Epitome Microarray covers 29,127 linear peptides printed in duplicate (58,254 peptide spots) as well as additional polio and HA control peptides.
  - Using the MEME tool (http://meme-suite.org/tools/meme), common motifs in the peptide top hits can be discovered.
  - Subsequent FIMO analysis (http://meme-suite.org/tools/fimo), translates hit motifs into possible cross-reactive antigens.

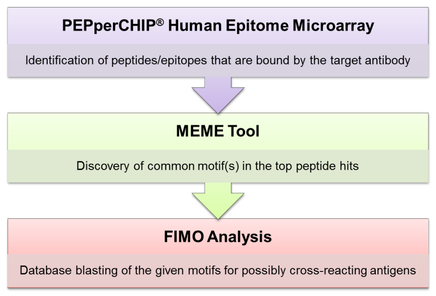
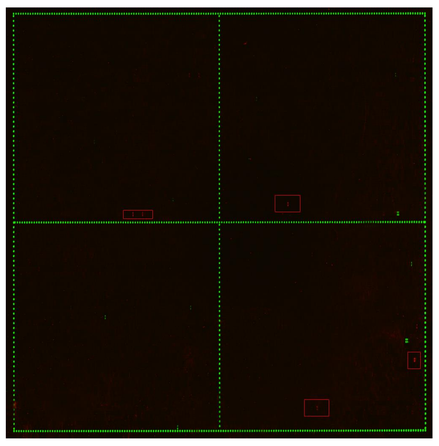


Figure 4: Worklflow of PEPperPRINT's three-step approach for antibody cross-reactivity analysis.

Results Antibody Cross-Reactivity Profiling

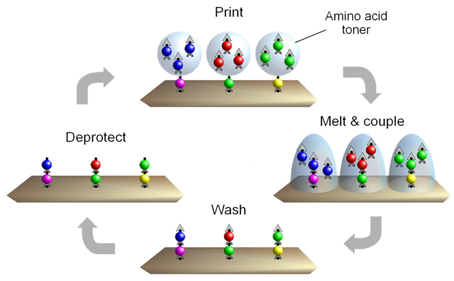
- PEPperCHIP® Human Epitome Microarray incubated with human mAb followed by staining with secondary and control antibodies resulted in few but clear peptide hits (red).
- Cross-reactions against epitopes of nucleoprotein, erythrocyte membrane-associated giant protein antigen 332 and dihydrolipoamide S-acetyltransferase.
- Cross-reactivity profile over 28,895 known human epitopes with database annotations.
- Identification of conserved and variable amino acid positions by MEME motif discovery.



Peptide	anti-c-Myc (chi9E10), 2 µg/ml	Epitope ID	Source Molecule Accession	Source Molecule Name	Source Organism ID	Source Organism Name
LGITAEDARLVSEIAMH	2.645,0	98874	127900	Nucleoprotein	11235	Measles virus strain Edmonston
EEVLVGEEKLVSEEIVT	2.535,0			Erythrocyte Membrane-Associated Giant Protein Antigen	5833	Plasmodium Falciparum
EVQEEGYLAKILVPE	1.960,5			Dihydrolipoamide S-Acetyltransferase (E2 Component Of	9606	Homo Sapiens
LAKILVPEGTDVP	1.559,0			Dihydrolipoamide S-Acetyltransferase (E2 Component Of	9606	Homo Sapiens
TAEDARLVSEIAMHTTE	1.050,5			Nucleoprotein	11235	Measles virus strain Edmonston
TYGRKLHLYSHPILGF	1.007,5			Polymerase	10407	Hepatitis B virus
ALVAEGIEAIVFRTL	923,0			Truncated Is1560 Transposase	1773	Mycobacterium tuberculosis
NGFLDVFTSFGGLVAE	748,0			Major Surface Protein V1se	224326	Borrelia Burgdorferi B31
FTSFGGLVAEAFGF	639,0			Major Surface Protein V1se	224326	Borrelia Burgdorferi B31
QYLVGERTVLAGQCYI	620,0			Muscarinic Acetylcholine Receptor M1	9606	Homo Sapiens
AQTQSLVYPF	593,0			Alpha-Casein Precursor	9913	Bos Taurus
VVSVVNTNVGLKFRQLL	547,5			Core Protein	10418	Hepatitis B virus subtype ayw
VLNPWDQVKR	545,5			Alpha-S2-Casein Precursor	9913	Bos Taurus
WLSLLVPFV	505,0			Large Surface Antigen	10407	Hepatitis B virus
GRSPRRRTPSPRRR	495,0			Core Protein	10407	Hepatitis B virus
GLSPTVWLSV	485,0	21139	128168864	Envelope Protein	10407	Hepatitis B virus
PSPRRRRSQSPRRRRR	475,5	55713	16930336	Core Protein	10407	Hepatitis B virus
WSEGEGAVFYRVDLHFI	468,0	119822	62094	110 Kd Polyprotein Precursor	11041	Rubella virus
GAAGTAAQAAVVRFQ	443,0	21976	15611010	10 Kda Culture Filtrate Antigen Esxb	1773	Mycobacterium tuberculosis
PWATLVAES	418,0	140582				
KYGGTEKYNGEEYLI	414,0	34437	116200	10 Kda Chaperonin	1773	Mycobacterium tuberculosis
RKLHLYSHPIILGFRKI	412,0	109568	4323200	Polymerase	10407	Hepatitis B virus
SSLRGF	408,0	156460				
MININIFMRESSRSFL	399,0	11711				
RRRSQSPRRRR	396,5	55713	16930336	Core Protein	10407	Hepatitis B virus
HYVLDTVVLLGALAN	383,0	25123	15609123	Probable Conserved Integral Membrane Protein	83332	Mycobacterium tuberculosis H37R
RIRRSILPYGDSMDRI	377,0	125882	146345399	Collagen Alpha-1(Xvii) Chain	9606	Homo Sapiens
WWARRRRRWRRWKRR	360,0	127897	5441235	Orf1	68887	Torque Teno Virus
FPGGGQIVGGVYVLPRR	357,0	150483	974351	Core Protein	356114	Hepatitis C Virus Genotype 3
LVAEEDER	352,5	7979	SRC279960	Genome Polyprotein	11103	Hepatitis C Virus
DVKFPGGGQI	332,0	10636	130461	Genome Polyprotein	11103	Hepatitis C Virus
GPSVFLF	331,0	107421	494350	Chain H, Three-Dimensional Structure Of A Human Immu	9606	Homo Sapiens
AIAEYERSAAVLVRYPF	329,5	193035	564602885	Peptidylarginine Deiminase	837	Porphyromonas Gingivalis
NPGLLRFLPQLSERL	324,0	179310	15609704	Hypothetical Protein	1773	Mycobacterium tuberculosis
FLPSDFFPSV	311,5	79531	116946	Capsid Protein	10418	Hepatitis B virus Subtype Ayw
ELGGKPALVPDRQVLYQ	311,0	20145	81992797	Genome Polyprotein	31647	Hepatitis C Virus Subtype 1B
VFCVQP	311,0	68440	130458	Genome Polyprotein	31647	Hepatitis C Virus Subtype 1B
QSLSFDSSNPEYFDGYW	309,0	176618	226694183	Integrin Alpha-Iib	9606	Homo Sapiens
AQLLTEFAI	308,0	4002	57117045	Ppe Family Protein	83332	Mycobacterium tuberculosis H37R
CLLCAYSIEF	301,5	7713	124757	Ovomucoid Precursor	9031	Gallus Gallus

Figure 5: PEPperCHIP® Human Epitome Microarray of human mAb anti-c-Myc. Interactions of anti-c-Myc with the database epitopes in red and well-defined frame of HA control peptides in green (left). Top 40 interactions of human mAb anti-c-Myc sorted by decreasing spot intensities. MEME analysis of the top 10 peptide hits of the PEPperCHIP® Human Epitome Microarray (right).

Technology



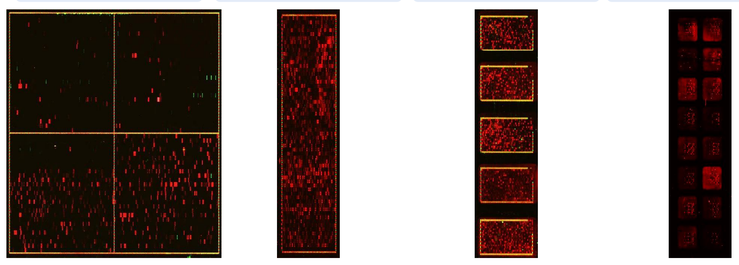
Benefits

- very low material consumption
- high spot density / high content (800 peptides/cm²)
- digital printing flexibility, fast production times
- high peptide quality with routine double couplings

- High-density PEPperCHIP® peptide microarrays are generated by digital laser printing on standard glass slides using a proprietary laser printer comprising 24 cartridges filled with individual amino acid toners.
- For array production, the amino acid toner are simultaneously printed with high precision on their respective positions on the glass slides. Peptide synthesis is initiated by melting the toner particles to 90 °C. Under these conditions, the amino acids are released and are available for coupling to the previous amino acid. The coupling cycle is completed by washing steps to remove excess building blocks and protecting groups. Finally, the array is ready for the next synthesis cycle with laser printing and coupling.
- The benefits of this technique are an unique flexibility in terms of peptide content, a high spot density with up to 11,000 features per chip and low material consumption enabling the generation of customized peptide array at reasonable costs.

PEPperCHIP® Peptide Microarray Platform

Discovery Format | Standard Format | Mapping Format | Multiplexed Format

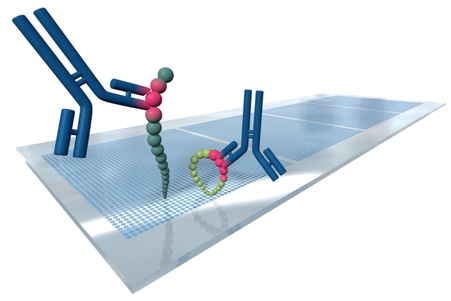


> 70,000 Peptides | ~ 11,000 Peptides | e.g. 5 x 1,200 Peptides | 16 x 200 Peptides

Antibody-peptide interactions are analyzed by immuno-type assays in a high-throughput fashion on peptide microarrays. Depending on the application, various microarray formats are available:

- **Discovery format:** approximately 70,000 individual peptides; suitable for screening of large, diverse epitope libraries or several full-length viral proteomes on a single chip; applied for epitope, biomarker and target binder discovery as well as antibody cross-reactivity analysis
- **Standard format:** covers approximately 11,000 individual peptides; routinely used for epitope mapping. Custom peptide microarrays or standard chips such as PEPperCHIP® Infectious Disease Epitope Microarray or the PEPperCHIP® Dengue Virus Type 1 Proteome Microarray among others
- **Mapping format:** several identical array copies on a single chip; ideal for parallel screening of multiple samples; used for epitope mapping of single protein antigens, detailed epitope characterization or biomarker validation
- **Multiplexed format:** up to 16 array copies on a single chip; ideal for assay development or hit validation studies with sample cohorts

Summary and Outlook



- **High Resolution Epitope Mapping of Rituximab** with cyclic constrained peptides reveals a conserved core motif EPANPSEK
- Cyclic constrained peptides on the peptide microarray enable the specific detection of the Rituximab binding signal.
- Loop sizes of cyclic constrained peptides influence binding signal intensity.
- Characterization of other, unknown epitopes of therapeutic and diagnostic antibodies can be conducted with high density peptide microarrays.

- **Antibody characterization and cross-reactivity analysis** of a human monoclonal antibody (anti-c-Myc (chi9E10)) is possible with the PEPperCHIP® Human Epitome Microarray - based three step approach.
- **Antibody Cross-Reactivity Profiling** reveals possible cross-reactivity with antigens containing the hit motif 'XLV(S/A/P)E'. Top candidates were based on the consensus motif HLVSE and assigned to DNA repair protein XRCC4, putative uncharacterized protein BVES-AS1, transmembrane protein 109 or choline O-acetyltransferase.
- Identification of other therapeutic and diagnostic antibodies as well as cross-reactivity analysis can be performed with PEPperCHIP® Human Epitome Microarray.