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

## Introduction

Antibodies are one of 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, as summarized in the recent Nature Feature *Reproducibility crisis: Blame it on the antibodies*:[1] The Human Protein Atlas consortium from Sweden has analyzed around 20,000 commercial research antibodies so far, and found that less than 50% can be effectively used for immunohistochemical tests. Researchers at Mount Sinai Hospital in Toronto, Canada, had been chasing a protein called CUZD1, a supposed diagnostic marker protein for pancreatic cancer. They bought a protein-detection kit comprising a CUZD1 specific antibody and wasted two years, $500,000 and thousands of patient samples before they realized that the antibody actually recognized a different cancer protein, CA125, but did not bind to CUZD1 at all.

These two examples of the Nature Feature strongly underline the urgent need for antibody validation and cross-reactivity testing. To address this topic, PEPperPRINT has developed a three-step approach based on the new PEPperCHIP® Human Epitome Microarray[2] (Fig. 1).

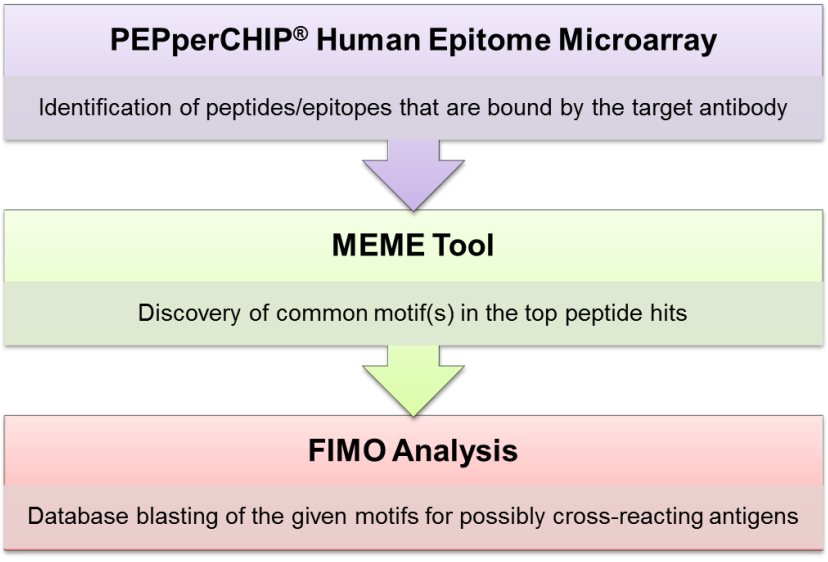



**Fig. 1:** Workflow of PEPperPRINT's three-step approach for antibody cross-reactivity analysis

In this application note, we describe the cross-reactivity analysis of a human monoclonal anti-c-Myc (chi9E10) antibody with the PEPperCHIP® Human Epitome Microarray and the three-step approach sketched in Fig. 1. The human monoclonal anti-c-Myc (chi9E10) antibody reacted with a number of peptides of the PEPperCHIP® Human Epitome Microarray with moderate to high signal-to-noise ratios. MEME analysis[3] of the top 10 peptide hits revealed a single consensus motif xLV(S/A/P)E that was further analyzed with the FIMO tool to identify cross-reactive human antigens containing the same motif.

## Results & Discussion

The PEPperCHIP® Human Epitome Microarray covers 29,127 linear peptides printed in duplicate (58,254 peptide spots) as well as additional polio and HA control peptides.[2] The peptide microarray content is based on all linear B-cell epitopes of the Immune Epitope Database[4] with the host "human“, and was further complemented by all epitopes of the most common vaccines. Therefore, the PEPperCHIP® Human Epitome Microarray is an ideal tool to screen for antibody responses against tens of thousands of different antigenic peptides that were described in literature and linked with antibody responses in human serum or plasma. Moreover, it enables straightforward access to scientific background information of each peptide hit by direct links to epitope entries in the Immune Epitope Database.

In a microarray pre-staining, PEPperCHIP® Human Epitome Microarray was initially incubated with the secondary antibody goat anti-human IgG (Fc) DyLight680 only to analyze background interactions with the printed database epitopes. Subsequently, the same microarray was incubated with human monoclonal antibody anti-c-Myc (chi9E10) with a concentration of 2 µg/ml followed by staining with the secondary antibody and control antibody mouse monoclonal anti-HA (12CA5) DyLight800. Data read-out was done with a LI-COR Odyssey Imaging System, and image analysis with PepSlide® Analyzer[5].

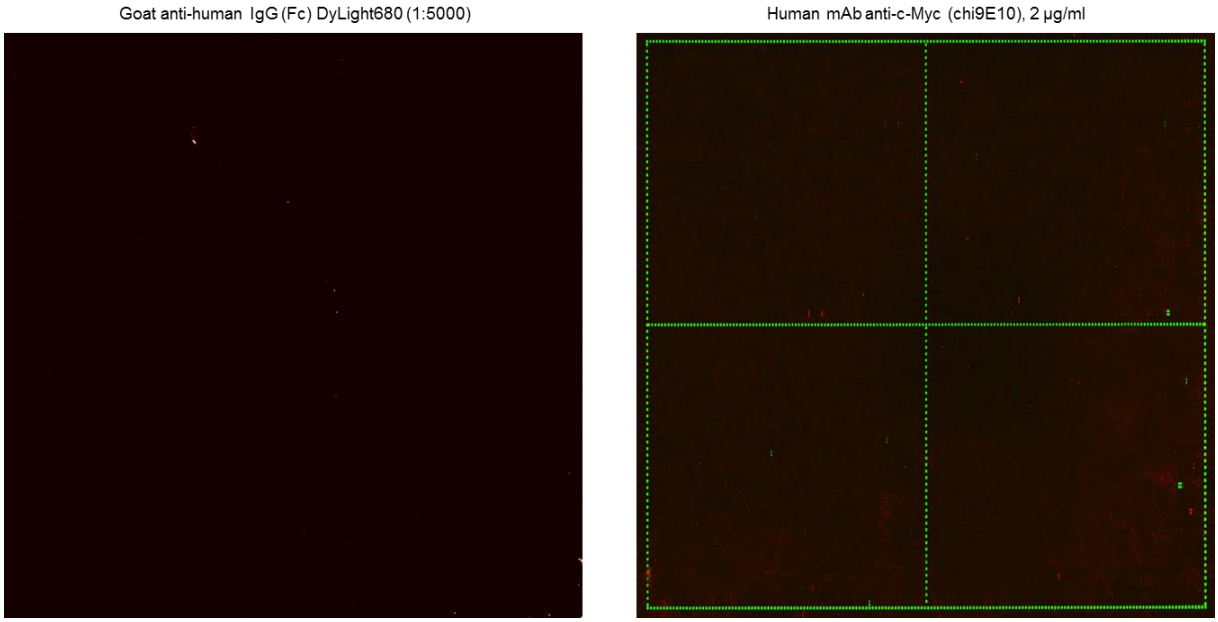



**Fig. 2:** Pre-staining with the PEPperCHIP® Human Epitome Microarray with the secondary antibody goat anti-human IgG (Fc) DyLight680 did not show any background interaction with the 29,127 database epitopes (left). Subsequent incubation of the same microarray with human monoclonal antibody anti-c-Myc (chi9E10) followed by staining with secondary and control antibodies highlighted few but clear interactions of human mAb anti-c-Myc (chi9E10) with the database epitopes in red as well as a well-defined frame of HA control peptides in green (right).

Pre-staining of the PEPperCHIP® Human Epitome Microarray with the secondary antibody only did not show background interactions with the database epitopes (Fig. 2, left). Incubation of the same microarray with human monoclonal antibody anti-c-Myc (chi9E10) was followed by staining with secondary and control antibodies and resulted in few but clear responses with moderate to high signal-to-noise ratios as well as a well-defined frame of HA control peptides (Fig. 2, right).

After data quantification, the top 40 interactions of human mAb anti-c-Myc (chi9E10) were sorted by decreasing spot intensities (Fig. 3). The main responses of the monoclonal antibody were directed against peptides of nucleoprotein of measles virus strain Edmonston, erythrocyte membrane-associated giant protein antigen 332 of *Plasmodium falciparum*, polymerase of hepatitis B virus and truncated Is1560 transposase of *Mycobacterium tuberculosis*. The strongest cross-reaction with a human epitope was found for dihydrolipoamide S-acetyltransferase peptides with the consensus motif LAKILVPE.

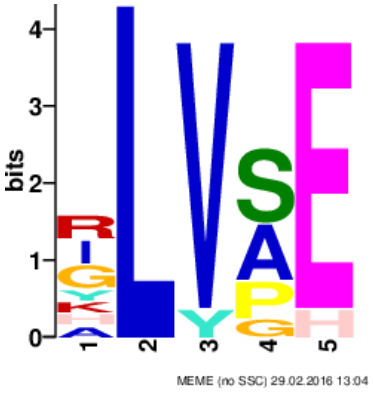
| Peptide | Human mAb anti-c-Myc (chi9E10), 2 µg/ml | Epitope ID | Source Molecule Accession | Source Molecule Name | Source Organism ID | Source Organism Name |
|---|---|---|---|---|---|---|
| LGITAEDARLVSEIAMH | 7.987,5 | 98874 | 127900 | Nucleoprotein | 11235 | Measles virus strain Edmonston |
| EEVVGEEKLVSEEIVT | 2.535,0 | 11927 | 13508497 | Erythrocyte Membrane-Associated Giant Protein Antigen | 5833 | Plasmodium Falciparum |
| EVQEEGYLAKILVPE | 1.960,5 | 125443 | 119587578 | Dihydrolipoamide S-Acetyltransferase (E2 Component Of | 9606 | Homo Sapiens |
| LAKILVPEGTRDVP | 1.559,0 | 125468 | 119587578 | Dihydrolipoamide S-Acetyltransferase (E2 Component Of | 9606 | Homo Sapiens |
| TAEDARLVSEIAMHTTE | 1.050,5 | 98874 | 127900 | Nucleoprotein | 11235 | Measles virus strain Edmonston |
| TYGRKLHLYSHPIILGF | 1.007,5 | 190568 | 4323200 | Polymerase | 10407 | Hepatitis B virus |
| ALVAEGIEAIVFRTL | 923,0 | 178535 | 15608176 | Truncated Is1560 Transposase | 1773 | Mycobacterium tuberculosis |
| NGFLDVFTSFGGLVAE | 748,0 | 6520 | 75352214 | Outer Surface Protein VIse | 224326 | Borrelia Burgdorferi B31 |
| FTSFGGLVAEAFGF | 639,0 | 18069 | 75352214 | Outer Surface Protein VIse | 224326 | Borrelia Burgdorferi B31 |
| QYLVGERTVLAGQCYI | 620,0 | 133331 | 113118 | Muscarinic Acetylcholine Receptor M1 | 9606 | Homo Sapiens |
| AQTQSLVYPF | 593,0 | 78152 | 162797 | Beta-Casein Precursor | 9913 | Bos Taurus |
| VVSYVNTNVGLKFRQLL | 547,5 | 53394 | 164509170 | Precore Protein | 10418 | Hepatitis B virus subtype ayw |
| VLNPWDQVKR | 545,5 | 95970 | 115654 | Alpha-S2-Casein Precursor | 9913 | Bos Taurus |
| WLSLLVPFV | 496,0 | 72794 | 15211906 | Large Surface Antigen | 10407 | Hepatitis B virus |
| GRSPRRRTPSPRRR | 495,0 | 55713 | 16930336 | Core Protein | 10407 | Hepatitis B virus |
| GLSPTVWLSV | 485,0 | 21139 | 128168864 | Envelope Protein | 10407 | Hepatitis B virus |
| PSPRRRRSQSPRRRR | 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 | | | | |
| KYGGTEIKYNGEEYLI | 414,0 | 34437 | 116200 | 10 Kda Chaperonin | 1773 | Mycobacterium tuberculosis |
| RKLHLYSHPIILGFRKI | 412,0 | 190568 | 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 |
| HVYLDTVVLLGALAN | 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 Immur | 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 |

**Fig. 3:** The top 40 peptide hits of human mAb anti-c-Myc (chi9E10) on the PEPperCHIP® Human Epitome Microarray were sorted by decreasing spot intensities. The second column shows the fluorescence intensities of each peptide with direct links to the corresponding Immune Epitope Database entries in the third column.

The top 10 peptide hits of human mAb anti-c-Myc (chi9E10) were uploaded to the MEME tool of the MEME Suite to discover common motifs in these peptides.[3] MEME represents motifs as position-dependent letter-probability matrices, which describe the probability of each possible letter at each position in the pattern. Analysis of the top 10 peptides of human mAb anti-c-Myc (chi9E10) resulted in a single consensus motif $^{1}$xLV(S/A/P)E$^{5}$ with a very high statistical significance of E = 1.0e-004 (Fig. 4, left). According to this MEME motif, amino acid $^{2}$L appeared to be essential for binding of human mAb anti-c-Myc (chi9E10), while amino acids $^{3}$V and $^{5}$E were highly conserved. Amino acid position 1 was rather variable, position 4 exhibited a clear preference for S, A and P.

The MEME consensus motif was uploaded into the FIMO tool ("Find Individual Motif Occurrences“), and processed in a protein database blast search with a focus on human proteins. The FIMO analysis resulted in 727 human proteins with the MEME motif $^{1}$xLV(S/A/P)E$^{5}$ or motif variants. Sorted by decreasing p-values and hence response probabilities, these proteins can be regarded as possible candidates for cross-reactions of human monoclonal antibody anti-c-Myc (chi9E10) (Fig. 4, right). The top FIMO 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. The expected response against human Myc proto-oncogene protein was also found among the database hits at position 175, albeit with a slightly less significant p-value.




| Sequence Name | Start | Stop | Score | p-Value | q-Value | Matched Sequence | Species | Database Entry |
|---|---|---|---|---|---|---|---|---|
| sp\|Q13426\|XRCC4_HUMAN | 9 | 13 | 15,49 | 6,71E-07 | 0,865 | HLVSE | Human | Q13426 |
| sp\|Q5T3Y7\|BVAS1_HUMAN | 40 | 44 | 15,49 | 6,71E-07 | 0,865 | HLVSE | Human | Q5T3Y7 |
| sp\|Q9BVC6\|TM109_HUMAN | 75 | 79 | 15,49 | 6,71E-07 | 0,865 | HLVSE | Human | Q9BVC6 |
| sp\|P28329\|CLAT_HUMAN | 152 | 156 | 15,49 | 6,71E-07 | 0,865 | HLVSE | Human | P28329 |
| sp\|Q9H0K6\|PUS7L_HUMAN | 208 | 212 | 15,49 | 6,71E-07 | 0,865 | HLVSE | Human | Q9H0K6 |
| sp\|Q8N2I9\|STK40_HUMAN | 222 | 226 | 15,49 | 6,71E-07 | 0,865 | HLVSE | Human | Q8N2I9 |
| sp\|P09917\|LOX5_HUMAN | 373 | 377 | 15,49 | 6,71E-07 | 0,865 | HLVSE | Human | P09917 |
| sp\|Q9UPU7\|TBD2B_HUMAN | 589 | 593 | 15,49 | 6,71E-07 | 0,865 | HLVSE | Human | Q9UPU7 |
| sp\|Q6N021\|TET2_HUMAN | 839 | 843 | 15,49 | 6,71E-07 | 0,865 | HLVSE | Human | Q6N021 |
| sp\|Q92673\|SORL_HUMAN | 914 | 918 | 15,49 | 6,71E-07 | 0,865 | HLVSE | Human | Q92673 |
| sp\|Q8WXI7\|MUC16_HUMAN | 915 | 919 | 15,49 | 6,71E-07 | 0,865 | HLVSE | Human | Q8WXI7 |
| sp\|Q6ZS81\|WDFY4_HUMAN | 2402 | 2406 | 15,49 | 6,71E-07 | 0,865 | HLVSE | Human | Q6ZS81 |
| sp\|Q6UN15\|FIP1_HUMAN | 8 | 12 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | Q6UN15 |
| sp\|Q8NBP5\|MFSD9_HUMAN | 14 | 18 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | Q8NBP5 |
| sp\|Q9Y5F3\|PCDB1_HUMAN | 65 | 69 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | Q9Y5F3 |
| sp\|Q8NCA9\|ZN784_HUMAN | 75 | 79 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | Q8NCA9 |
| sp\|Q6NT89\|TRNP1_HUMAN | 118 | 122 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | Q6NT89 |
| sp\|Q13516\|OLIG2_HUMAN | 171 | 175 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | Q13516 |
| sp\|Q6XPS3\|TPTE2_HUMAN | 203 | 207 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | Q6XPS3 |
| sp\|O00515\|LAD1_HUMAN | 254 | 258 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | O00515 |
| sp\|Q5TZA2\|CROCC_HUMAN | 308 | 312 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | Q5TZA2 |
| sp\|Q99489\|OXDD_HUMAN | 323 | 327 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | Q99489 |
| sp\|Q9NRW7\|VPS45_HUMAN | 340 | 344 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | Q9NRW7 |
| sp\|Q15276\|RABE1_HUMAN | 508 | 512 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | Q15276 |
| sp\|P43304\|GPDM_HUMAN | 525 | 529 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | P43304 |
| sp\|Q8TER5\|ARH40_HUMAN | 1088 | 1092 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | Q8TER5 |
| sp\|P78357\|CNTP1_HUMAN | 1255 | 1259 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | P78357 |
| sp\|Q96L96\|ALPK3_HUMAN | 1607 | 1611 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | Q96L96 |
| sp\|P46939\|UTRO_HUMAN | 1692 | 1696 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | P46939 |
| sp\|Q8WZ42\|TITIN_HUMAN | 2841 | 2845 | 15,20 | 2,31E-06 | 0,865 | RLVSE | Human | Q8WZ42 |

**Fig. 4:** MEME analysis of the top 10 peptide hits of the PEPperCHIP® Human Epitome Microarray resulted in a single consensus motif [1]xLV(S/A/P)E[5] with a very high statistical significance (left). Amino acid [2]L was found to be essential for binding of human mAb anti-c-Myc (chi9E10), amino acids [3]V and [5]E were also highly conserved. Amino acid position 1 was rather variable, while position 4 exhibited a clear preference for amino acids S, A and P. FIMO analysis of this motif in Swissprot resulted in 727 human proteins as possible targets for antibody cross-reactivity sorted by decreasing response probabilities. The table shows the top 30 hits with highest statistical significances based on consensus motifs HLVSE and RLVSE (right).

## Conclusion

Using our three-step cross-reactivity analysis based on the PEPperCHIP® Human Epitome Microarray and the MEME Suite with the MEME motif and FIMO tools, we could identify a common peptide motif [1]xLV(S/A/P)E[5] that is recognized by test antibody anti-c-Myc (chi9E10). A database blast search with this consensus motif resulted in a probability-dependent list of human proteins as possible candidates for antibody cross-reactions. Interestingly, the c-Myc antigen was also found among the candidate proteins, albeit with a lower statistical significance than the top hits. On the PEPperCHIP® Human Epitome Microarray, we further identified a clear cross-reaction with dihydrolipoamide S-acetyltransferase peptides with the consensus motif LAKILVPE with the xLVPE stretch.

The results of the PEPperCHIP® Human Epitome Microarray and the MEME/FIMO analysis enable a cross-reactivity evaluation of a given antibody with respect to its application. A more detailed analysis of the identified common motif can be done by a PEPperMAP® Epitope Substitution Scan to identify tolerated amino acid exchanges and essential amino acid positions.[6] Alternatively, selected cross-reactions can be validated by ELISA or in Western Blot analysis.

[1] http://www.nature.com/news/reproducibility-crisis-blame-it-on-the-antibodies-1.17586
[2] http://www.pepperprint.com/products/human-epitome-microarray/
[3] http://meme-suite.org/
[4] http://www.iedb.org/
[5] http://www.pepperprint.com/high-content-peptide-microarrays/
[6] http://www.pepperprint.com/applications/epitope-substitution-scan/