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Epitope Discovery

Antibody Cross-Reactivity Profiling of Human Monoclonal Antibody anti-c-Myc (chi9E10) with the PEPperCHIP® Human Epitome Microarray

PEPperPRINT GmbH
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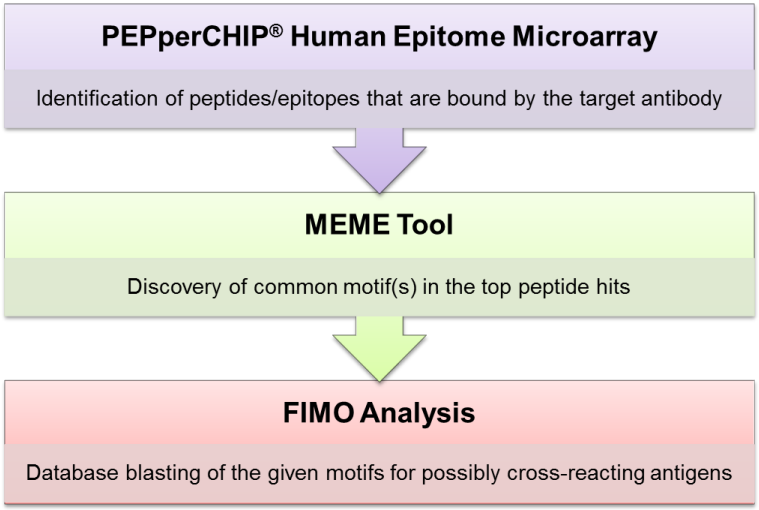
Introduction

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*: 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: The PEPperCHIP® Human Epitome Microarray with 29,127 different peptides covers all linear B-cell epitopes of the Immune Epitope Database with the host “human”. The microarray is, hence, 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. The three-step approach is further complemented by bioinformatics analysis of the top cross-reactions of an antibody using the MEME and FIMO tools of the MEME Suite for motif discovery and correlation with protein databases.



This application note describes the cross-reactivity analysis of a human monoclonal anti-c-Myc (chi9E10) antibody with the aforementioned three-step approach. The human monoclonal antibody reacted with a number of peptides of the PEPperCHIP® Human Epitome Microarray with moderate to high signal-to-noise ratios. MEME analysis of the top peptide hits revealed a common motif xLV(S/A/P)E that was further analyzed with the FIMO tool to identify cross-reactive human antigens containing the same motif.

Material and Methods

Microarray Content:	The screening was done with a PEPperCHIP® Human Epitome Microarray with 29,127 linear peptides printed in duplicate (58,254 peptide spots). The peptide microarray content was based on all linear B-cell epitopes of the Immune Epitope Database with the host "human“, and was further complement by all epitopes of the most common vaccines. The PEPperCHIP® Human Epitome Microarray contained additional polio (KEVPALTAVETGAT, 772 spots) and HA (YPYDVPDYAG, 775 spots) control peptides as specified in the peptide map of the microarray data file.
Sample:	Human monoclonal antibody anti-c-Myc (chi9E10)
Washing Buffer:	PBS, pH 7.4 with 0.05% Tween 20 (3x1 min after each assay)
Blocking Buffer:	Rockland blocking buffer MB-070 (30 min before the first assay)
Incubation Buffer:	PBS, pH 7.4 with 0.05% Tween 20 and 10% Rockland blocking buffer
Assay Conditions:	Antibody concentration of 2 µg/ml in incubation buffer; incubation for 16 h at 4°C and shaking at 140 rpm
Secondary Antibody:	Goat anti-human IgG (Fc) DyLight680 (1:5000); 45 min staining in incubation buffer at RT
Control Antibody:	Monoclonal anti-HA (12CA5) DyLigh800 (1:2000); 45 min staining in incubation buffer at RT
Scanner:	LI-COR Odyssey Imaging System; scanning offset 0.65 mm, resolution 21 µm, scanning intensities of 7/7 (red = 700 nm/green = 800 nm)
Microarray Data:	Microarray Data mAb anti-c-Myc (chi9E10).xlsx
Microarray ID:	0001050_04V

Experimental and Data Analysis

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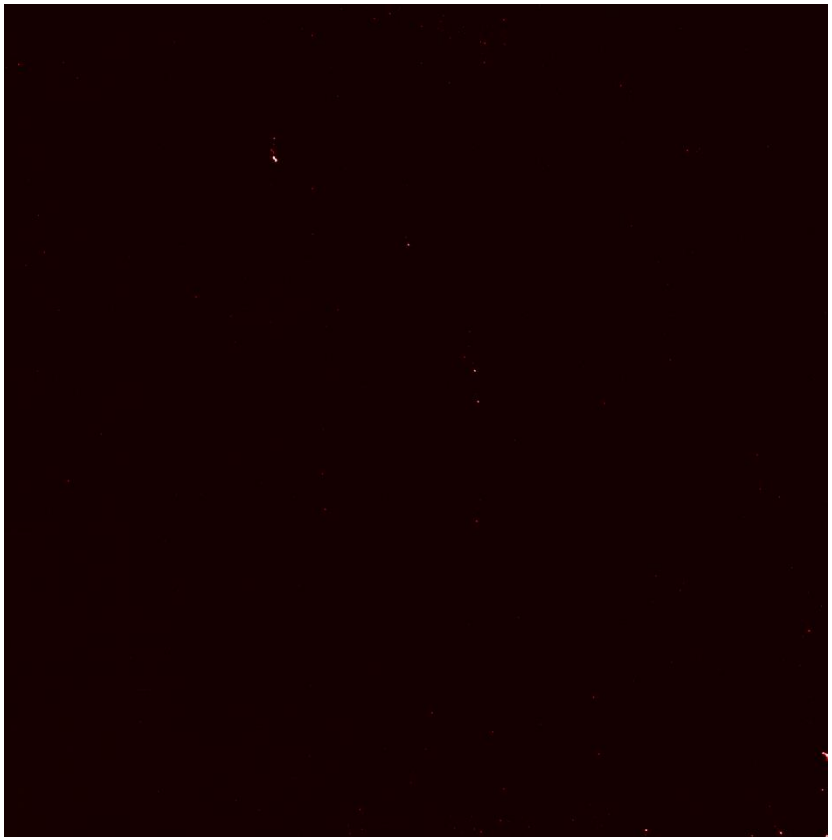
After 15 min pre-swelling in standard buffer and 30 min in blocking buffer, the PEPperCHIP® Human Epitome Microarray was initially incubated with the secondary antibody goat anti-human IgG (Fc) DyLight680 at a dilution of 1:5000 to analyze background interactions with the printed database epitopes. Subsequent incubation of the PEPperCHIP® Human Epitome Microarray was done with human monoclonal antibody anti-c-Myc (chi9E10) at a concentration of 2 µg/ml in incubation buffer. After washing, the microarray was stained with the secondary antibody goat anti-human IgG (Fc) DyLight680 (1:2000) and control antibody mouse monoclonal anti-HA (12CA5) DyLigh800 (1:2000) followed by data read-out with a LI-COR Odyssey Imaging System at scanning intensities of 7/7 (red/green). The additional HA peptides framing the PEPperCHIP® Human Epitome Microarray were simultaneously stained as internal quality control to confirm the assay quality and the peptide microarray integrity (scanning intensities: 7/7, red/green).

Quantification of spot intensities and peptide annotation were based on the 16-bit gray scale tiff file that exhibit a higher dynamic range than the 24-bit colorized tiff files; microarray image analysis was done with PepSlide® Analyzer and summarized in Excel file *Microarray Data mAb anti-c-Myc (chi9E10).xlsx*. A software algorithm breaks down fluorescence intensities of each spot into raw, foreground and background signal (see “Raw Data” tab), and calculates the standard deviation of median foreground intensities (see “Mapping Summary” tab). Spots with a deviation of 40% were zeroed to yield corrected averaged foreground intensities. Based on corrected averaged median foreground intensities, an intensity map was generated and binders in the peptide map were highlighted by an intensity color code with red for high and white for low spot intensities.

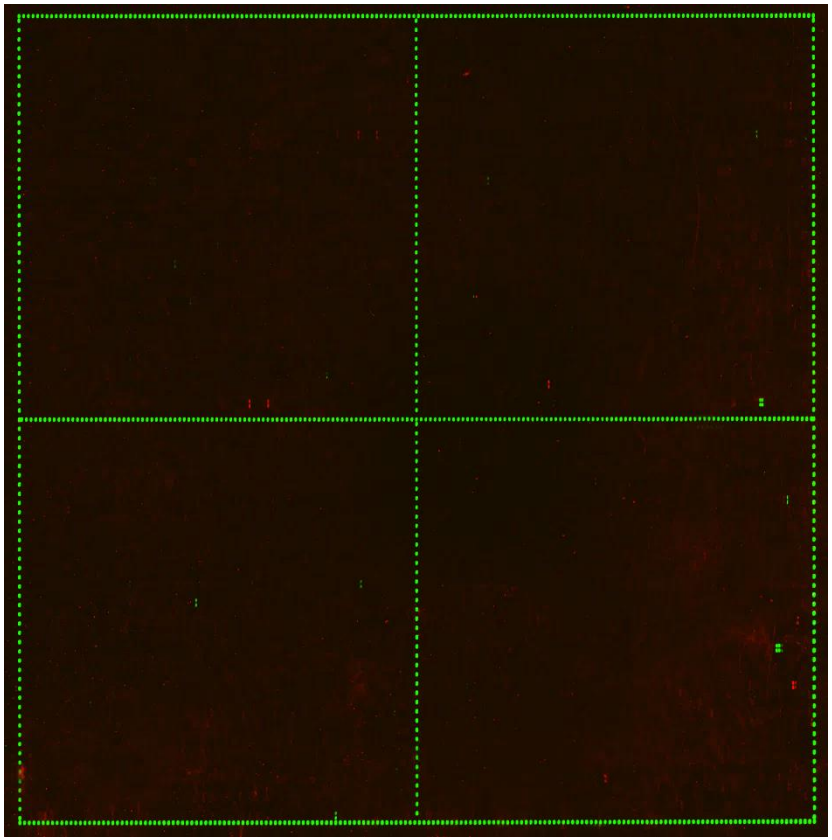
To identify the top responses of the antibody sample, the averaged and corrected intensity values were sorted by decreasing spot intensities (see “Top Peptides” tab). We further plotted averaged spot intensities of the assay with human monoclonal antibody anti-c-Myc (chi9E10) against the microarray content from left on top to right on bottom of the PEPperCHIP® Human Epitome Microarray to visualize overall spot intensities and signal-to-noise ratios (see “Intensity Plot” tab). The main responses were annotated next to the corresponding signal in the graph. The intensity plots were correlated with peptide and intensity maps as well as with visual inspection of the microarray scan to identify peptides that were recognized by the human monoclonal antibody.

Microarray Scans

Goat anti-human IgG (Fc) DyLight680 (1:5000)

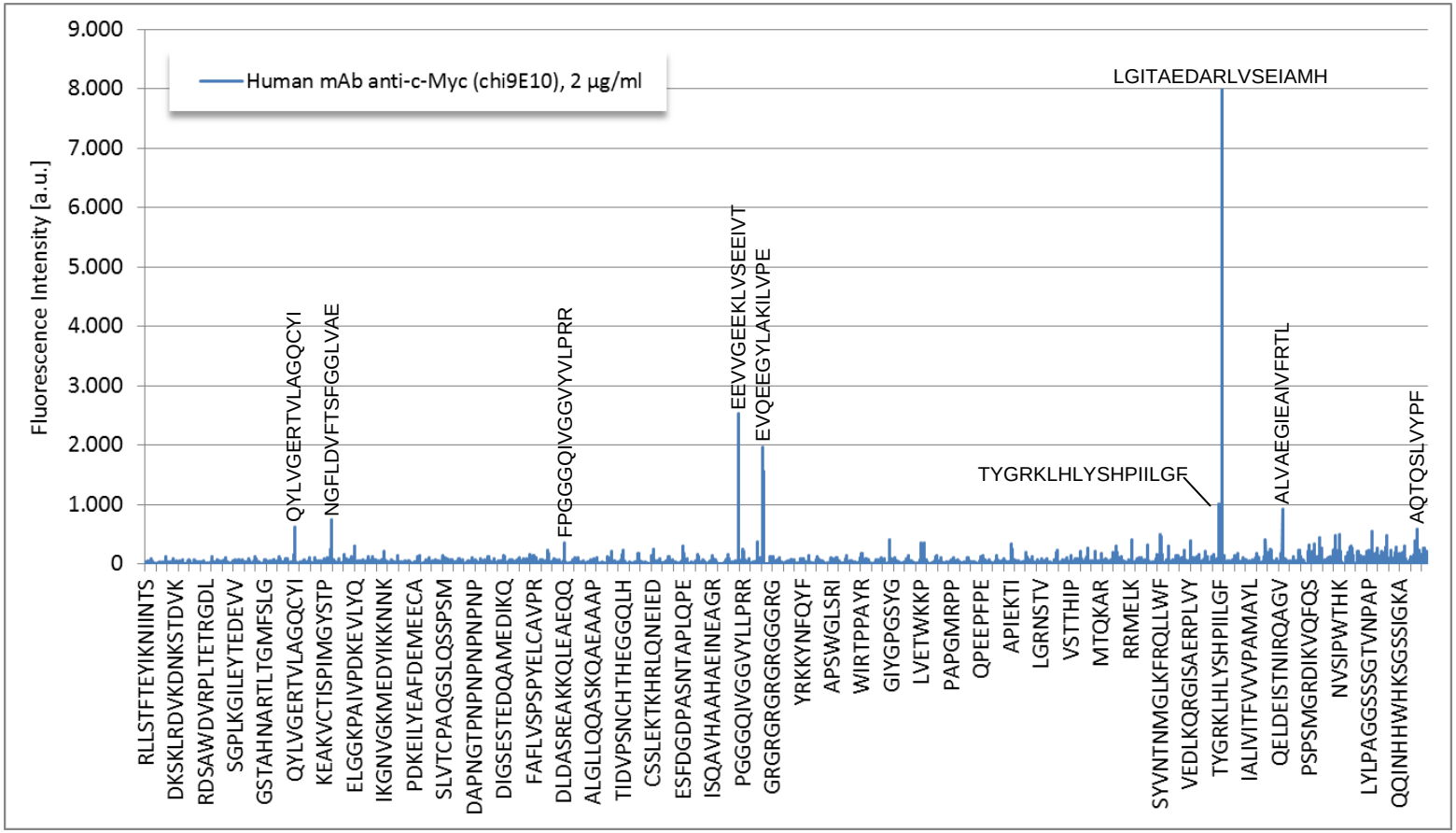


Human mAb anti-c-Myc (chi9E10), 2 µg/ml



- incubation of PEPperCHIP® Human Epitome Microarray 0001050_04V with secondary antibody goat anti-human IgG (Fc) DyLight680 (1:2000) followed by read out at scanning intensities of 7/7 (red/green; left) did not show any background interaction with the data base epitopes; data quantification with PepSlide® Analyzer was hence omitted
- incubation with human monoclonal antibody anti-c-Myc (chi9E10) at a concentration of 2 µg/ml was followed by staining with secondary and control antibodies as well as microarray data read out (right); we observed 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

Intensity Plot anti-c-Myc (chi9E10)



- data quantification was followed by generation of an intensity plot for the response of human mAb anti-c-Myc (chi9E10) assayed at a concentration of 2 µg/ml from left on top to right on bottom of the PEPperCHIP® Human Epitome Microarray
- main responses with moderate to high spot intensities between 1,000 and 8,000 fluorescence units as well as a number of weaker interactions with less than 1,000 units; the strongest responses were found for peptides LGITAEDARLVSEIAMH and TAEDARLVSEIAMHTTE of Measles virus strain Edmonston, peptide EEVVGEEKLVSEEIVT of Plasmodium falciparum as well as for two peptides with the consensus motif LAKILVPE of dihydrolipoamide S-acetyltransferase

Top Peptides anti-c-Myc (chi9E10)

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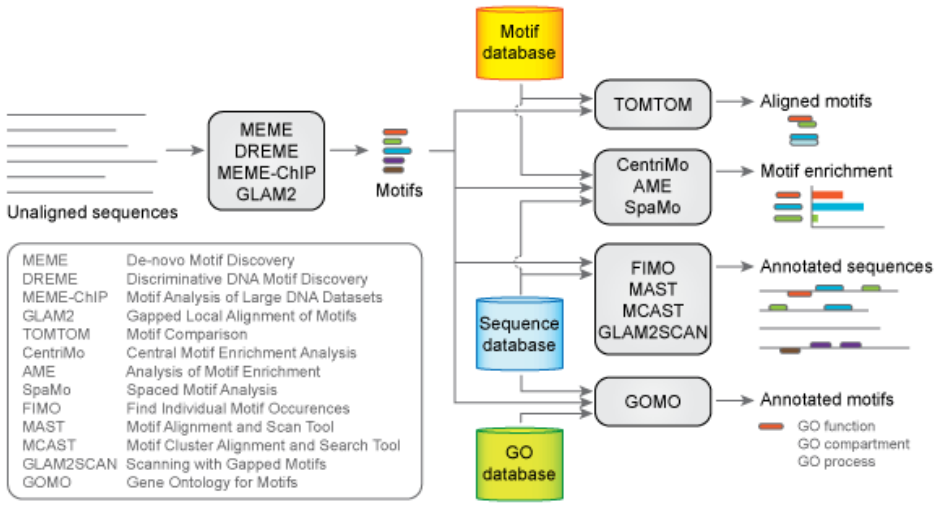
Peptide	Human mAb anti-c-Myc (chi9E10), 2 µg/ml	Epitope ID	Source Molecule Accession	Source Molecule Name	Source Organism ID	Source Organism Name
LGITAEDARLVSEIAMH	7.987,5	98874	127900	Nucleoprotein	11235	Measles virus strain Edmonston
EEVVGEEKLVSEEIVT	2.535,0	11927	13508497	Erythrocyte Membrane-Associated 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 Vlse	224326	Borrelia Burgdorferi B31
FTSFGGLVAEAFGF	639,0	18069	75352214	Outer Surface Protein Vlse	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 H37Rv
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 H37Rv
CLLCAYSIEF	301,5	7713	124757	Ovomucoid Precursor	9031	Gallus Gallus

- data quantification of the PEPperCHIP® Human Epitome Microarray was followed by removal of clear assay artifacts and spots with a deviation of more than 40%;
- the top 40 interactions of human mAb anti-c-Myc (chi9E10) were sorted by decreasing spot intensities (see also tab “Top Peptides” of Excel file *Microarray Data mAb anti-c-Myc (chi9E10).xlsx*)

MEME/FIMO Bioinformatic Hit Analysis



To identify consensus motifs of the antibody cross-reactions, we performed MEME/FIMO bioinformatic analyses of the top peptide hits by using the MEME Suite covering motif-based sequence analysis tools.[1]

For this purpose, the top 10 cross-reactions of human mAb anti-c-Myc (chi9E10) were uploaded to the MEME tool for discovering motifs in a group of related DNA or protein sequences. MEME represents motifs as position-dependent letter-probability matrices which describe the probability of each possible letter at each position in the pattern. Individual MEME motifs do not contain gaps.

The MEME pre-settings were a maximum of one motif each sequence and of max. 3 different motifs, as well as a minimum motif length of 5 amino acids. The E-value corresponds to the statistical significance of a consensus motif with the given log likelihood ratio (or higher), and with the same width and site count, that one would find in a similarly sized set of random sequences. An E-value of 1 would be expected for the identification of a certain motif in a set of random peptides by chance; decreasing E-values correlate with increasing statistical significance. Therefore a significance threshold of 1.0e-001 was defined for this analysis.

With MEME E-values below 1.0e-001, the resulting consensus motifs were submitted to the FIMO tool ("Find Individual Motif Occurrences“). FIMO is a tool for searching biological sequence databases for sequences that contain one or more known motifs. FIMO searches a sequence database for occurrences of user provided motifs, treating each motif independently. The program uses a dynamic programming algorithm to convert log-odds scores into p-values, assuming a zero-order background model. The p-values for each motif are then converted to q-values following the method of Benjamini and Hochberg (where "q-value" is defined as the minimal false discovery rate at which a given motif occurrence is deemed significant). The program reports all motif occurrences that receive p-values smaller than a specified threshold.

[1] http://meme.nbcr.net/meme/intro.html/

MEME/FIMO Bioinformatic Analysis

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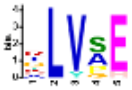
Discovered Motifs

	Logo	E-value	Sites	Width	More	Submit/Download
1.		1.0e-004	10	5		
2.		3.0e+001	2	5		
3.		1.5e+002	2	5		

Stopped because requested number of motifs (3) found.

MEME analysis of the top 10 peptides resulted in a single 5 amino acid consensus motif with a very high statistical significance of E = 1.0e-004. 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 3 exhibited a clear preference for S, A and P. The two other motifs were statistically not relevant

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

FIMO analysis of the main MEME motif in Swissprot resulted in 727 database hits of human antigens. Sorted by increasing p-values, the top motifs were based on epitope stretches HLVSE, RLVSE, YLVSE, ILVSE or HLVPE (see also tab “FIMO Analysis” of Excel file *Microarray Data mAb anti-c-Myc (chi9E10).xlsx*). Each hit was linked with the corresponding UniProt database entry. The top responses were 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, albeit with a slightly less significant p-value.

Discussion and Conclusion

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The antibody cross-reactivity profiling of human monoclonal antibody anti-c-Myc (chi9E10) was based on a PEPperCHIP® Human Epitome Microarray with all linear B-cell epitopes of the Immune Epitope Database with the host "human". The peptide content covered 29,127 different epitope-derived peptides printed in duplicate as well as additional polio (772 spots) and HA (775 spots) control peptides. The PEPperCHIP® Human Epitome Microarray was incubated with the human monoclonal antibody at a concentration of 2 µg/ml in incubation buffer followed by staining with secondary antibody goat anti-human IgG (Fc) DyLight680 and with control antibody mouse monoclonal anti-HA (12CA5) DyLigh800 as well as read-out with a LI-COR Odyssey Imaging System. Quantification of spot intensities and peptide annotation were done with PepSlide® Analyzer.

The pre-staining of the PEPperCHIP® Human Epitome Microarrays with the **secondary antibody goat anti-human IgG (Fc) DyLight680** did not show any background interaction with the 29,127 different database peptides. Subsequent incubation the PEPperCHIP® Human Epitome Microarray with **human mAb anti-c-Myc (chi9E10)** resulted in few but clear interactions with moderate to high signal-to-noise ratios. 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.

In accordance with the wild type epitope EQKLISEEDL of human mAb anti-c-Myc (chi9E10), MEME analysis showed that the cross-reactions were based on a statistically significant 5 amino acid motif xLV(S/A/P)E: Except for a conserved exchange of V by I, the MEME motif clearly resembled the KLISE stretch of the wild type sequence. A FIMO analysis of the 5 amino acid motif xLV(S/A/P)E resulted in 727 database hits of human antigens as candidates for cross-reactions of human monoclonal antibody anti-c-Myc (chi9E10), what was attributed to the rather short conserved core motif of the MEME analysis. Sorted by increasing p-values, the top motifs were based on epitope stretches HLVSE, RLVSE, YLVSE, ILVSE or HLVPE 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, albeit with a slightly less significant p-value.

Discussion and Conclusion

The results of the PEPperCHIP® Human Epitome Microarray and the MEME/FIMO analysis now enable a cross-reactivity evaluation of a given antibody with respect to its application and the biological relevance of the candidate proteins: An intracellular target in a different organ is likely negligible, while a frequent extracellular receptor may play a more important role for cross-reactions. For an in-depth analysis of conserved and variable amino acid positions of wild type peptide EQKLISEEDL and/or of the top cross-reactive peptide LGITAEDARLVSEIAMH, we recommend full PEPperMAP® Full Substitution Scans with exchanges of all amino acid positions with the 20 main amino acids (see also application note "PEPperMAP® Full Substitution Scan of SMN Epitope"). That way we can identify the actual epitope lengths and further screen for possible cross-reactions based on bioinformatic analysis of the conserved and validated core motif. On request PEPperPRINT can also validate epitope candidates by ELISA tests with synthetic peptides, or by EC_{50} determination of antibody-epitope interactions.

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