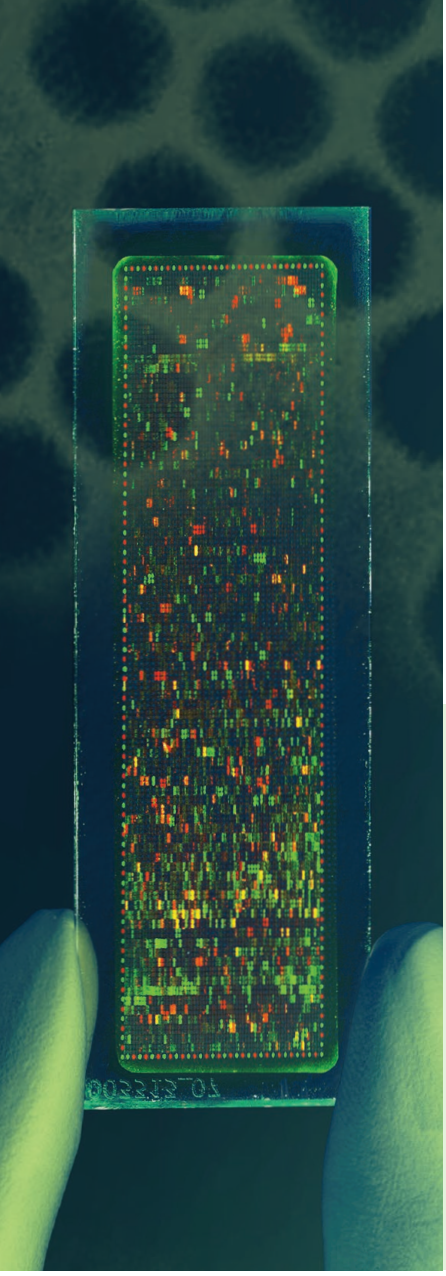

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Application Note

High-resolution linear and conformational epitope mapping of anti-AAV antibodies with PEPperCHIP® Pan-AAV Capsid Protein Microarrays

ABSTRACT

Adeno-associated virus (AAV) vectors are a key platform for *in vivo* gene delivery. Clinical trials are ongoing for the treatment of a variety of diseases, and so far, two AAV-based gene therapies are approved.

Antibodies are essential tools in life sciences with a multitude of applications in medicine and biomedical research. For all applications, the specificity of a certain antibody is of utmost importance to generate reliable and robust data. For AAV research, a wide range of anti-AAV antibodies is available, which can be used e.g. for the detection and classification of AAV capsid proteins and virus particles. This application note describes the use of PEPperCHIP® Pan-AAV Capsid Protein Microarrays for anti-AAV antibody validation and in-depth epitope analysis (Figure 1). We applied this approach to determine the epitope specificity of commercially available anti-AAV antibodies.

INTRODUCTION

AAVs are small (~ 25 nm in diameter), non-enveloped and non-pathogenic viruses belonging to the family of *Parvoviridae* (1). As reflected in the genus name *Dependoparvovirus*, the replication of AAVs depend on the presence of a helper virus such as adenovirus or herpes virus. Thus far, at least 13 naturally occurring primate serotypes have been described (2, 3), possessing different tissue tropisms. AAVs infect a wide range of different hosts including humans, in which infection is asymptomatic. The seroprevalence in the human population ranges from 15-90%, depending on the AAV serotype and the study cohort (1).

In recent years, AAVs emerged as a leading gene delivery vehicle for gene therapies. Properties like their broad tissue tropism, the low cytotoxicity and immunogenicity, the non-pathogenicity, the ability to enable long-term *in vivo* transgene expression and the possibility to engineer cargo and capsid make AAVs a promising *in vivo* gene delivery tool (3, 4).

In the AAV field, research is ongoing e.g. for AAV capsid and genetic cassette engineering and the optimization of large-scale production (4). Antibodies against AAVs are utilized in assay formats such as ELISA, immunohistochemistry, immunofluorescence, immunoprecipitation and Western Blot. In general, highly specific antibodies are a prerequisite to generate reliable results, emphasizing the need for appropriate validation methods in order to determine antibody specificity and any off-target binding. For AAV antibodies, this is in particular important in the context of serotype-specific antibodies, since the AAV capsid proteins share a high sequence homology across serotypes (5, 6).

High-density peptide microarrays are not only a powerful tool to simultaneously screen tens of thousands of peptides against serum antibodies in a high-throughput context (7) but also to conduct high-resolution epitope mappings of antibodies on the amino acid level (8-10).

In this case study, we performed high-resolution linear and conformational epitope mapping of three commercially

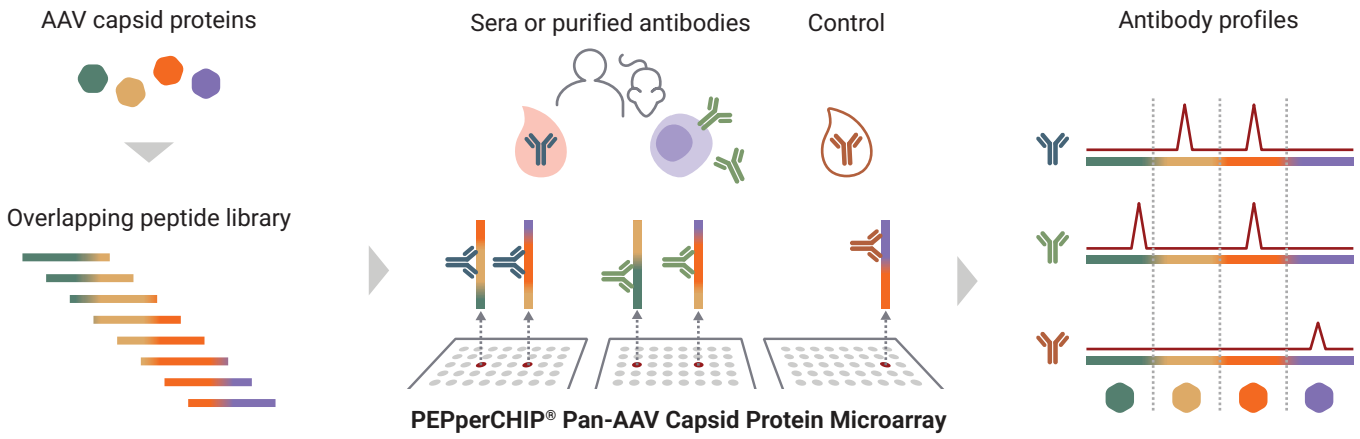

Figure 1. Analysis of antibody responses against different AAV serotypes on the epitope level. PEPperCHIP® Pan-AAV Capsid Protein Microarrays covering the capsid proteins of 15 wild-type AAVs as overlapping peptides can either be used to analyze epitope-specific responses of serum antibodies, or to perform an high-resolution epitope mapping of anti-AAV antibodies. This enables the monitoring of antibody specificities against capsid proteins derived from different AAV serotypes. In the present study, we used PEPperCHIP® Pan-AAV Capsid Protein Microarrays to determine the epitope specificity of selected anti-AAV antibodies. After incubation of the microarrays with the antibody samples, peptide-bound antibodies were subsequently detected with fluorescently labeled secondary antibodies. Image acquisition and data quantification result in the identification of the antibody epitope, and accordingly in the determination of the antibody specificity.

available anti-AAV antibodies using PEPperCHIP® Pan-AAV Capsid Protein Microarrays covering the capsid proteins (VP1) of AAV serotypes AAV1, AAV2, AAV3b, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.10, AAVpo.1, AAV10, AAV11, AAV12 and AAV13 converted into >5,500 different overlapping peptides. This approach allows not only the determination of the antibody epitope, but also to uncover potential cross-reactivity towards other AAV serotypes.

RESULTS AND DISCUSSION

In this study, we mapped three commercially available anti-AAV antibodies (antibodies-online GmbH, Germany; Table 1) on PEPperCHIP® Pan-AAV Capsid Protein Microarrays covering the capsid proteins (VP1) of 15 AAV serotypes as overlapping linear or conformational peptides. The resulting linear peptide microarrays comprise 5,519 different peptides printed in duplicate, the conformational peptide microarrays 5,534 different peptides printed in duplicate, respectively. Both linear and conformational PEPperCHIP® Pan-AAV Capsid Protein Microarrays were framed by poliovirus VP1 protein (11-13) and influenza hemagglutinin (HA)-derived control peptides.

Table 1. Anti-AAV antibodies used for epitope analysis (antibodies-online GmbH, Germany)

# Antibody	Target	Immunogen	Host	Clonality	Isotype
1	AAV4	NFTKLRPTNFSNC	rabbit	polyclonal	IgG
2	AAV VP1/VP2/VP3	TPWGYFDFNRFHC	rabbit	polyclonal	IgG
3	AAV5	NSQPANPGTTATC	rabbit	polyclonal	IgG

The rabbit polyclonal anti-AAV antibodies were diluted to a final concentration of 1 µg/ml in PBS with 0.005% Tween 20 and 10% Rockland blocking buffer MB-070 and incubated overnight at 4°C. Detection was done using a secondary goat anti-rabbit IgG (Fc) DyLight680 antibody. Fluorescence read-out was performed with an Innopsys InnoScan® 710-IR Microarray Scanner. Quantification of spot intensities was done with PepSlide® Analyzer based on the scanner 16-bit gray-scale images.

For all polyclonal rabbit anti-AAV antibodies, we observed clear and strong responses on both linear and conformational PEPperCHIP® Pan-AAV Capsid Protein Microarrays (Figure 2), already with antibody concentrations of 1 µg/ml.

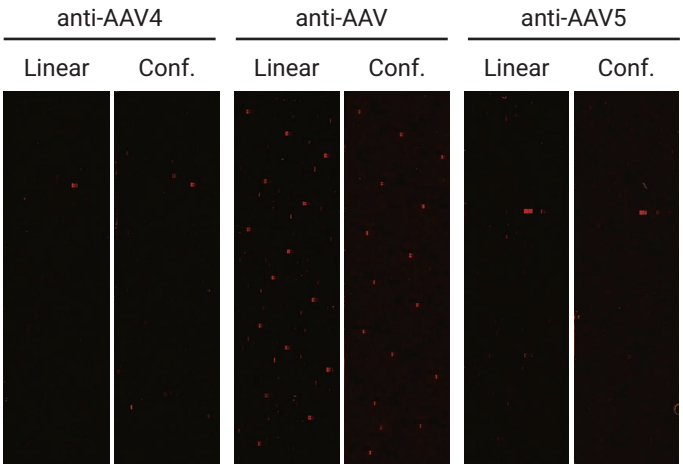

Figure 2. Microarray images showing the epitope patterns of the different anti-AAV antibodies on linear and conformational PEPperCHIP® Pan-AAV Capsid Protein Microarrays. The corresponding anti-AAV antibodies (antibodies-online GmbH, Germany) were diluted to a concentration of 1 µg/ml and incubated over night at 4°C. The next day, staining was performed using a secondary anti-rabbit IgG DyLight680 antibody.

The rabbit anti-AAV4 and anti-AAV5 antibodies exhibited a defined epitope stretch, whereas with the anti-AAV antibody showed several epitope stretches across the entire PEPperCHIP® Pan-AAV Capsid Protein Microarrays (Figure 2). This is in good agreement with the peptide sequences used for antibody generation (Table 1): The peptide selected to immunize animals for the generation of the anti-AAV antibody is highly conserved between different AAV serotypes. Vice versa, the peptides used to generate the anti-AAV4 and anti-AAV5 antibodies were derived from less conserved capsid regions with a higher difference between the AAV serotypes (Figure 3).

Data quantification highlighted only marginal differences of the intensity levels when responses of the anti-AAV antibodies were compared on linear versus conformational PEPperCHIP® Pan-AAV Capsid Protein Microarrays (Figure 4).

Further epitope mapping analyses allowed the annotation of the consensus motifs for the anti-AAV antibodies analyzed in this study (Figure 4 and Table 2). Again, we detected no differences in epitope recognition on linear versus conformational arrays with respect to the determined consensus motifs.

In parallel to the epitope mapping analysis, the PEPperCHIP® Pan-AAV Capsid Protein Microarray can also be used to analyze the specificity of anti-AAV antibodies towards the capsid sequences of 15 different AAV serotypes covered by the microarrays. As shown in Figure 4, the rabbit polyclonal anti-AAV antibodies exhibited only minor cross-reactions. For the anti-AAV4 antibody, we identified a single off-target interaction with cyclic-constrained peptide MATAGPSDGDFSN.

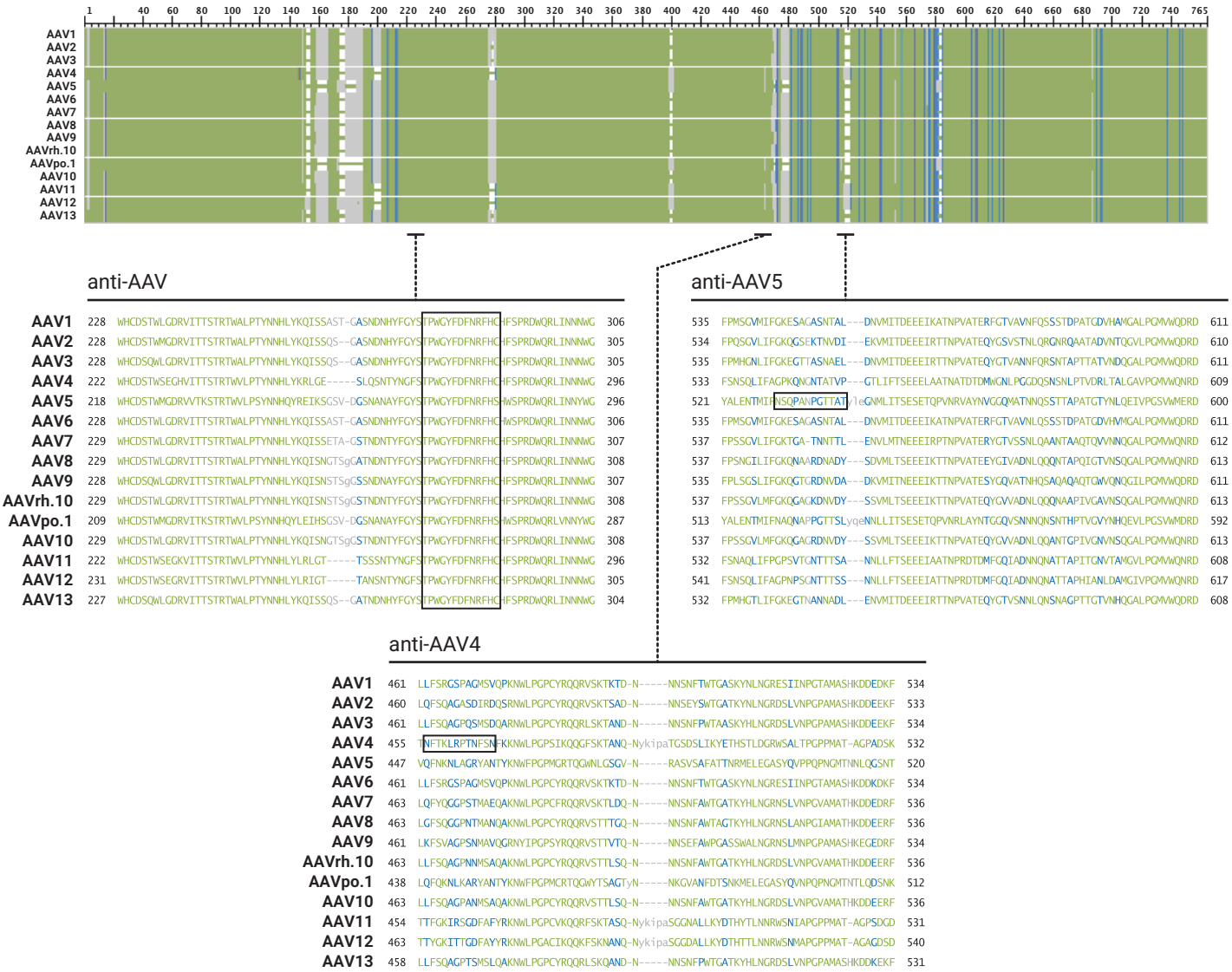

Figure 3. Sequence alignment of the capsid proteins covered by the PEPperCHIP® Pan-AAV Capsid Protein Microarrays. Multiple sequence alignment was performed using COBALT: Multiple Alignment Tool (14). **Upper graph: Overview of the alignment results.** (The capsid proteins of 15 AAV serotypes covered by the PEPperCHIP® Pan-AAV Capsid Protein Microarrays exhibit predominantly highly conserved positions (green) and few regions with a lower degree of sequence conservation (blue). Gray indicates gaps in the sequences added by the alignment program. **Lower graphs: Sequence regions in which the peptides for antibody production are located.** The anti-AAV antibody recognizes a highly conserved epitope, whereas the anti-AAV4 and anti-AAV5 antibodies were raised against serotype-specific sequences.

Table 2. Determined consensus motifs of the analyzed rabbit anti-AAV antibodies

# Antibody	Target	Immunogen	Determined consensus motif
1	AAV4	NFTKLRPTNFSNC	NFTKLRPTN
2	AAV VP1/VP2/VP3	TPWGYFDFNRFHC	(T)PWGYFDFN(R)
3	AAV5	NSQPANPGTTATC	QPANP

To investigate possible cross-reactivities in more detail, we further tested a 10-fold higher antibody concentration. Figure 5 shows the comparative analysis for the rabbit anti-AAV4 antibody. Increasing the antibody concentration resulted in weak additional interactions with peptides with the basic consensus motif (Q/A)PA(K/R)K. Although this consensus motif was not part of the original peptide antigen, it exhibited a minor sequence homology with the actual epitope QPANP based on the short QPA motif. This minor sequence homology possibly in combination with ionic effects due to the additional basic amino acids was likely responsible for the weak cross-reactions of the rabbit anti-AAV4 antibody at higher antibody concentrations.

CONCLUSION

This application note presents a workflow using our proprietary peptide microarray technology to perform an antibody epitope mapping analysis and to simultaneously determine the specificity of antibodies. The PEPperCHIP® Pan-AAV Capsid Protein Microarray enables the analysis of antibody responses towards the capsid protein (VP1) of 15 different AAV serotypes, and is an ideal tool to evaluate the specificity of anti-AAV antibodies. Moreover, the PEPperCHIP® Pan-AAV Capsid Protein Microarrays can be used to get a detailed understanding of antibodies against AAV vectors in patient sera. This is of utmost importance, as anti-AAV antibodies may affect the success of using AAVs in gene therapy.

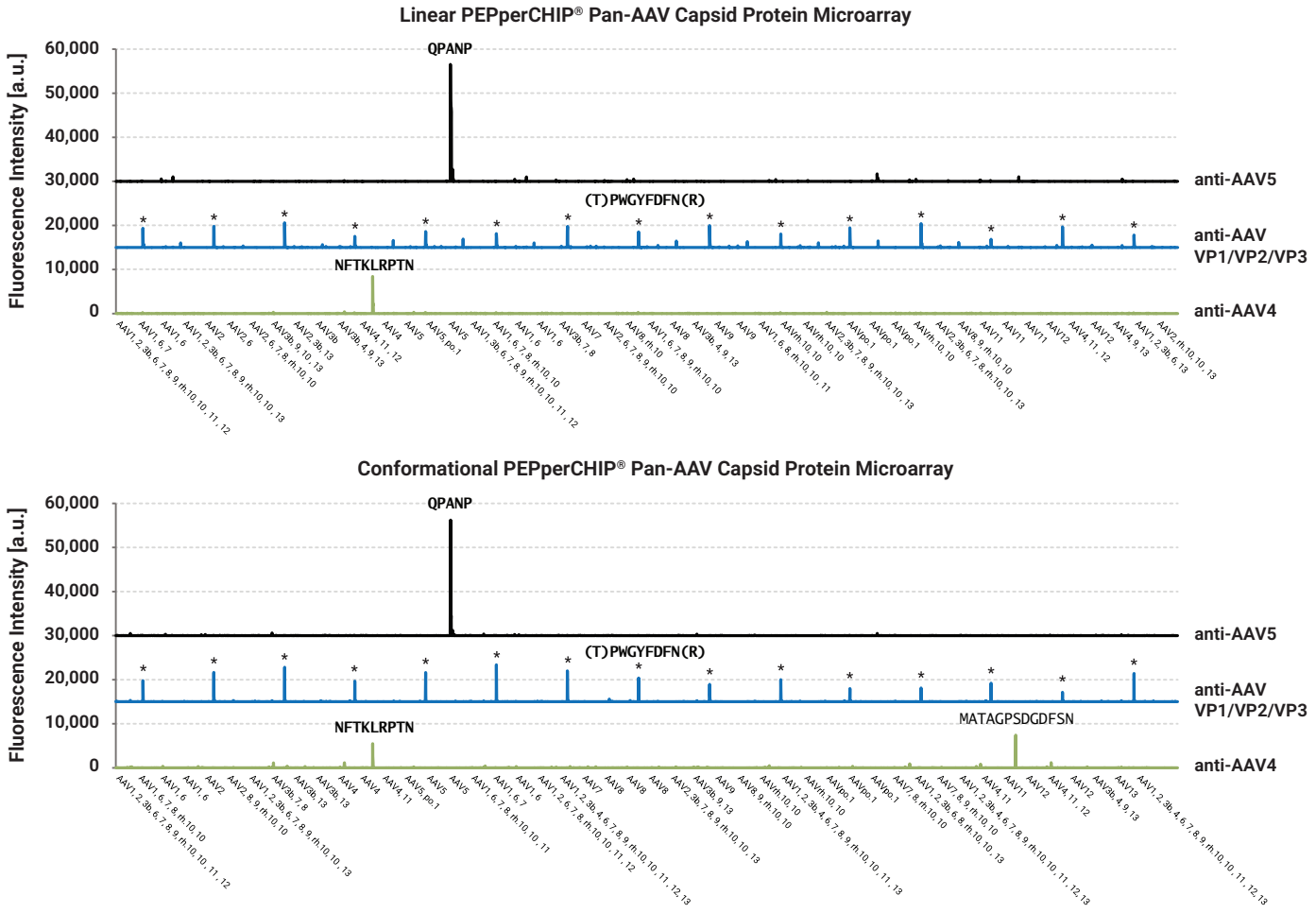

Figure 4. Epitope mapping of anti-AAV antibodies on linear and conformational PEPperCHIP® Pan-AAV Capsid Protein Microarrays. Linear (top) and conformational (bottom) PEPperCHIP® Pan-AAV Capsid Protein Microarrays were incubated with anti-AAV4, anti-AAV and anti-AAV5 antibodies (1 µg/ml), respectively. Detection was performed using a secondary anti-rabbit IgG DyLight680 antibody. Intensity plots show the raw fluorescence intensity units (y-axis) plotted against overlapping peptides derived from capsid proteins (VP1) of 15 AAV serotypes (x-axis). For a better comparison, the reactivity profile of the three tested anti-AAV antibodies on linear and conformational peptide microarrays, respectively, were summarized in one graph each. For that, responses of anti-AAV and anti-AAV5 were leveled. Respective antibody epitopes i.e. consensus motifs were annotated in the graph. Green line = epitope pattern of anti-AAV4 antibody; blue line = epitope pattern of anti-AAV antibody; black line = epitope pattern of anti-AAV5 antibody. The stars indicate that the epitope detected by the anti-AAV antibody is present in different AAV serotypes (see Figure 3).

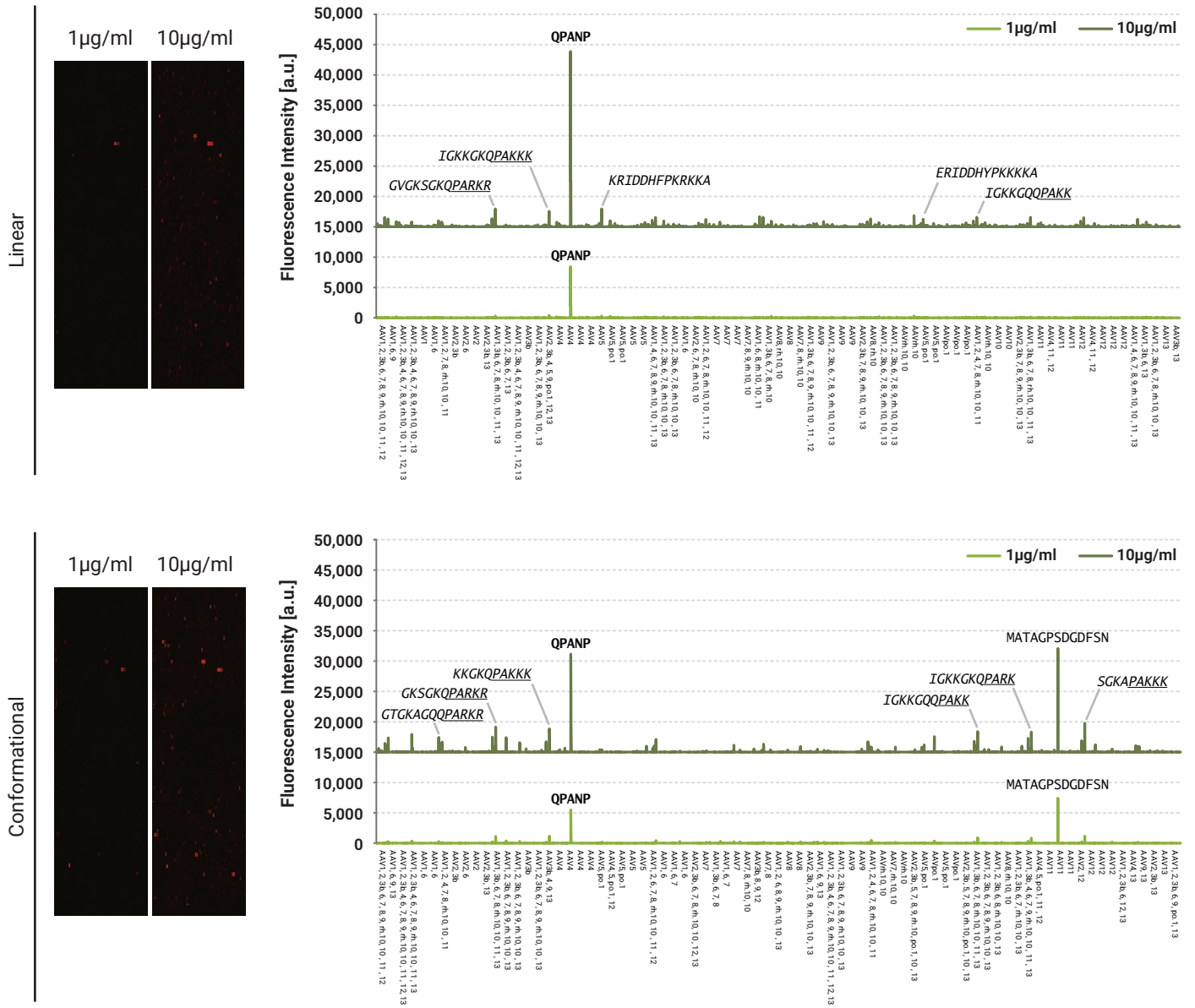

Figure 5. Cross-reactivity analysis of anti-AAV4 antibody on linear and conformational PEPperCHIP® Pan-AAV Capsid Protein Microarrays. Linear (upper graph) and conformational (lower graph) PEPperCHIP® Pan-AAV Capsid Protein Microarrays were incubated with anti-AAV4 (AA 456-467) at a concentration of 1 µg/ml and 10 µg/ml, respectively. Detection was performed using a secondary anti-rabbit IgG DyLight680 antibody. Intensity plots show the raw fluorescence intensity units (y-axis) plotted against overlapping peptides derived from capsid proteins (VP1) of 15 AAV serotypes (x-axis). For a better comparison, the reactivity profile of the two tested anti-AAV4 antibody concentrations on linear and conformational peptide microarrays, respectively, were summarized in one graph each. For that, responses of anti-AAV4 at 10 µg/ml were leveled. The antibody epitope i.e. consensus motif QPANP as determined by epitope mapping is shown (see also Figure 4). At higher antibody concentrations, weak interactions with peptides with the basic motif (Q/A)PA(K/R)K were detected (peptide sequences are indicated in the graphs). Green line = epitope pattern of anti-AAV4 antibody at 1 µg/ml; dark green line = epitope pattern of anti-AAV4 antibody at 10 µg/ml.

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