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Application Note

Epitope Discovery and Validation of Vaccination-Induced Antibody Responses with PEPperCHIP® Peptide Microarrays and a Bead-Based Multiplex Assay

ABSTRACT

This application note describes the use of PEPperCHIP® Peptide Microarrays for epitope discovery combined with a multiplexed bead-based assay for epitope validation (Figure 1). We applied this approach to unravel antibody responses on the epitope level induced upon COVID-19 (coronavirus disease 2019) vaccination.

For this, we screened sera from individuals pre- and post- COVID-19 vaccination on peptide microarrays covering the sequences of spike proteins derived from seven pathogenic coronaviruses as overlapping peptides. The multiplexed high-resolution epitope mapping revealed immunogenic epitopes identified in post-vaccination sera, which were further validated by a multiplexed bead-based assay (MAGPIX®-System, Luminex).

INTRODUCTION

The worldwide ongoing transmission of COVID-19 is a major global health concern. The course of the disease ranges from non-symptomatic to mild symptoms such as fever and cough to severe cases with pneumonia, acute respiratory distress and potentially death[1-3]. Neutralizing antibody responses are crucial to combat SARS-CoV-2 infection[4,5] with the spike glycoprotein being the major target antigen emphasizing the role of spike protein as the leading vaccine candidate. Since early 2020, enormous efforts have been made for the development of safe and effective COVID-19 vaccines, and remarkably, the first COVID-19 vaccines were already approved in mid to late 2020[6]. As of February 21st 2022, over 10 billion vaccine doses have been administered globally[7].

Analyzing vaccine-induced immune responses is important to determine the immunogenicity of a given vaccine i.e. to define and understand the type of the induced immune response and the magnitude over time. With respect to humoral responses to COVID-19 vaccination, both the induction and ultimately the titers of different classes of virus-specific antibodies are of particular interest. This also includes the circulation of neutralizing antibodies as essential surrogate marker of protective immunity[8]. Most studies use full-length spike protein or domains thereof for the analysis of virus-specific antibodies [e.g. 9-15]. However, analyses on vaccine-elicited antibody responses on the epitope level enabling the mapping of antibody interactions at amino acid resolution are limited[16, 17].

High-density peptide microarrays are a powerful tool to simultaneously screen tens of thousands of peptides against serum antibodies in a high-throughput context[18]. Antibody responses to linear and conformational epitopes can rapidly be analyzed yielding high and low immunogenic epitopes with amino acid resolution.

Multiplexed bead-based technologies such as the Luminex® xMAP® platform emerged as valuable approach for the simultaneous screening of up to 500 targets such as proteins, peptides or nucleic acids, immobilized on

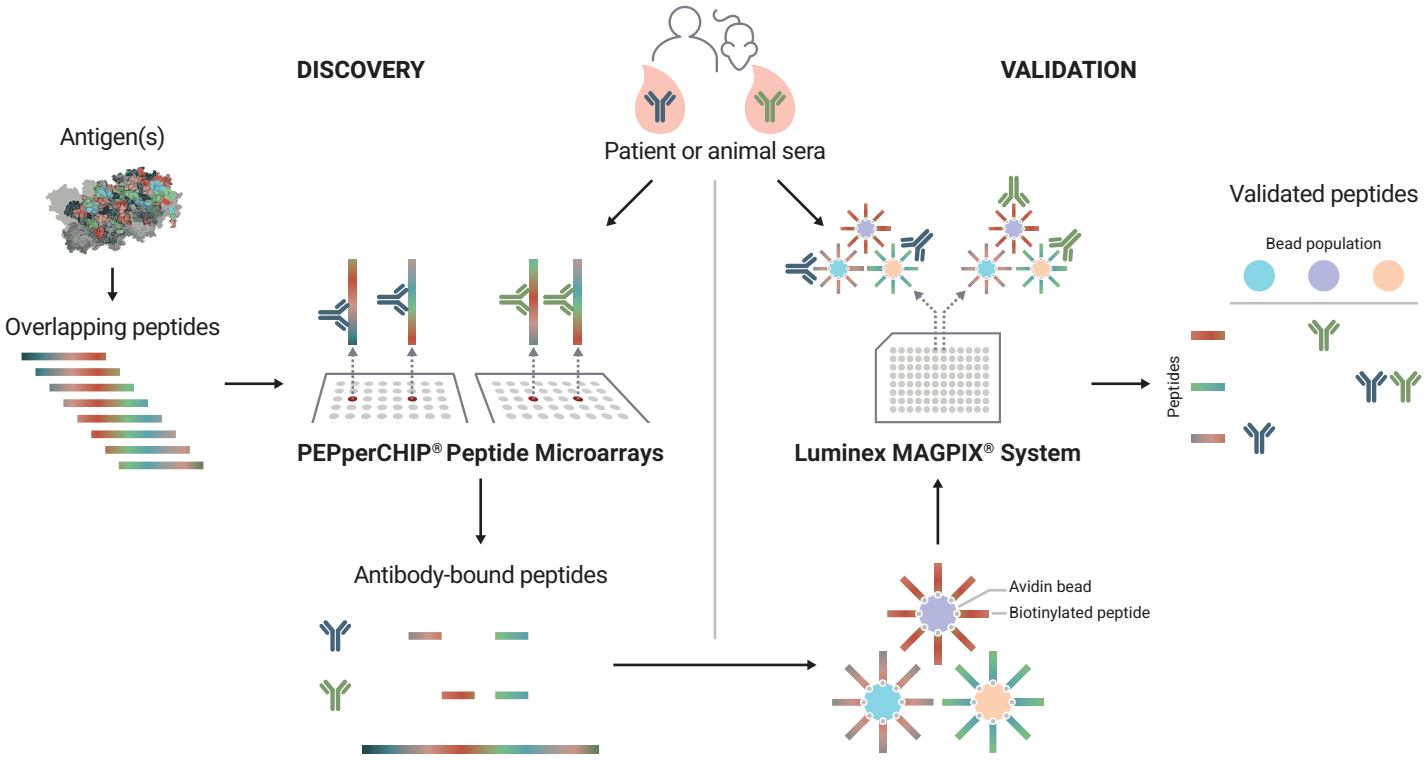

Figure 1. Workflow for epitope discovery and validation. Serum samples are initially screened with PEPperCHIP® Peptide Microarrays displaying peptide libraries of up to 70,000 peptides. Identified hit candidates are further validated applying a multiplexed bead-based immunoassay.

color-coded beads[19]. The beads are ideally suited for analyzing serological antibody responses to immobilized antigens or epitope peptides.

This application note describes an experimental workflow for exploring humoral responses on epitope level using peptide microarrays and subsequent validation of antibody reactivities against identified epitopes/peptides applying a multiplexed bead-based technology.

RESULTS AND DISCUSSION

In this study, we mapped the humoral responses of COVID-19 vaccine recipients. To determine the vaccine-elicited antibodies, we initially screened sera before and after COVID-19 vaccination on PEPperCHIP® Pan-Corona Spike Protein Microarrays covering the spike proteins of seven coronaviruses as overlapping linear 15 amino acid peptides (Figure 2). This allows not only the mapping of antibody responses towards SARS-CoV-2 spike protein, but also the analysis of potential cross-reactive antibodies recognizing spike protein sequences from other coronaviruses. The resulting peptide microarrays comprise 4,564 peptides printed in duplicates and were framed by poliovirus VP1 protein[20-22] and influenza hemagglutinin (HA)-derived control peptides.

For the subsequent epitope fingerprint analysis, sera of six individuals before and after the second COVID-19 vaccination (2-4 weeks after the 2nd dose) were used. The study cohort included the following vaccination regimens: 4 out of 6 subjects received a mRNA-based vaccination (Spikevax® or Comirnaty®), one a vector-based vaccination (Vaxzevria®) and one underwent a heterologous vaccination with a vector-based vaccine (Vaxzevria®) for the first and a mRNA-based vaccine (Comirnaty®) for the second immunization.

PEPperCHIP® Pan-Corona Spike Protein Microarrays were initially incubated with the secondary goat anti-human IgG (Fc) DyLight680 and goat anti-human IgA (alpha chain) DyLight800 antibodies to control for background interactions of the detection antibodies. Here, no background interactions were observed.

Subsequently, sera were diluted 1:200 and incubated overnight at 4°C. Detection was done using the secondary goat anti-human IgG (Fc) DyLight680 and goat anti-human IgA (alpha chain) DyLight800 antibodies. A mouse monoclonal anti-HA DyLight800 was added to stain the frame of HA control peptides. Fluorescence readout was performed using a LI-COR Odyssey Imaging System, and 16-bit grey-scale images were analyzed with the PepSlide® Analyzer software.

A comparison of the reactivity profile of sera taken before and after the 2nd vaccination highlighted epitope-specific IgG antibody responses against SARS-CoV-2 spike protein post vaccination (Figure 2 & Figure 3). For IgA, no relevant epitope recognition was observed. Therefore, further analysis was focused only on IgG responses.

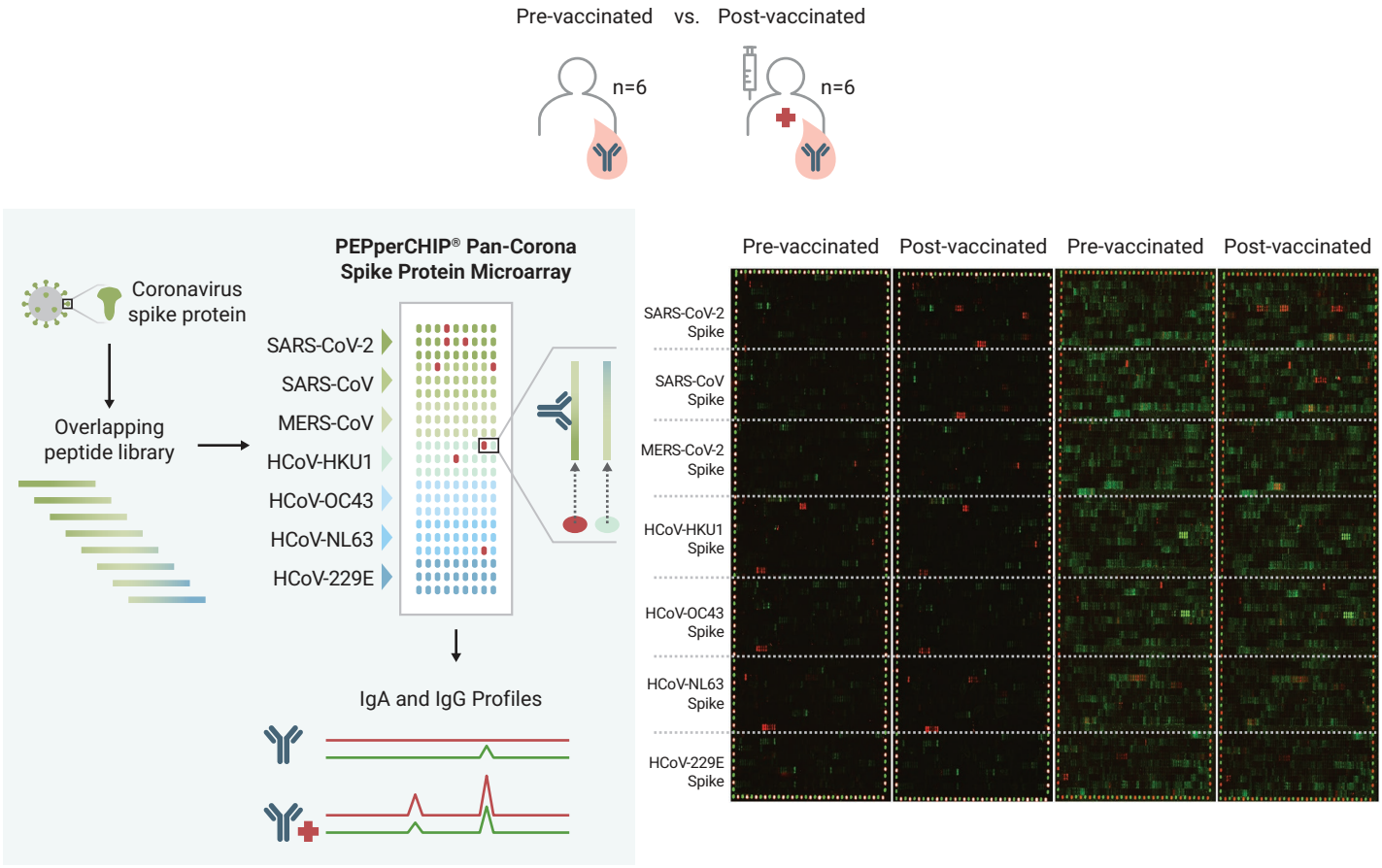

Figure 2. Epitope mapping of COVID-19 vaccine-induced antibody responses using PEPperCHIP® Pan-Corona Spike Protein Microarrays. Left side: Overview about the experimental workflow. Pre- and post-vaccination sera were screened on PEPperCHIP® Pan-Corona Spike Protein Microarrays comprising spike proteins from seven coronaviruses as overlapping peptides. A dual isotype read-out was performed to analyze IgG and IgA responses simultaneously. **Right side: Representative microarray scans from two vaccine recipients** (before and after the second COVID-19 vaccination). Sera were diluted 1:200 and incubated over night at 4°C. The next day, detection was performed using secondary anti-human IgG DyLight680 and anti-human IgA DyLight800 antibodies. Mouse monoclonal anti-HA DyLight800 was added to stain the HA peptide control frame. Red spots= IgG responses, green spots = IgA responses. For the control frame: Red spots = Polio responses from serum; green spots = HA signal. The microarray layout with the position of the overlapping spike peptides of the respective coronavirus on the microarray is indicated in the experimental workflow.

In addition to epitope-specific responses against SARS-CoV-2 spike after vaccination, cross-reactive IgG responses against the other corona spike protein sequences were also detected - presumably due to homologous consensus motifs between SARS-CoV-2, SARS-CoV and MERS-CoV spike proteins[23]. As expected, the screening further revealed pre-existing IgG responses against spike protein sequences derived from the four endemic human coronaviruses with no major differences in epitope recognition between pre- and post-vaccination sera (Figure 2 & Figure 3A).

The in-depth analysis of the IgG responses resulted in six major immunogenic epitopes, which were observed in at least two post-vaccination sera (Figure 3B). With the available sample set, the epitope pattern seemed to be widely independent on the type of the received vaccine (i.e. mRNA- or vector-based). Among the mapped epitopes, five out of six showed up in ≥ 67% of vaccine recipients, while two subjects recognized the epitope FDEDDSEPV.

Interestingly, in COVID-19 infection, it has been shown that antibodies directed against peptides with the core sequences KFLPFQQ and EELDKYF (Figure 3B) have neutralizing activity[24,25].

To validate the epitopes identified by the peptide microarray screening, a bead-based multiplex immunoassay was applied (Luminex xMAP® technology). For this, peptides with a C-terminal biotin-tag (Table 1) were coupled to paramagnetic MagPlex®-Avidin Microspheres (Luminex).

Coupling of peptides to MagPlex®-Avidin Microspheres (see Table 1 for respective bead regions) was carried out using 5 µg peptide per 10^6 beads. As internal control, positive control beads were prepared by coupling a common poliovirus peptide (Table 1), originating from an immunodominant region of the viral VP1 protein and shown to be highly reactive for human sera[20-22]. Next, peptide-coupled microspheres were combined (Figure 1) and incubated with diluted sera (1:100) in duplicate overnight at 4°C. Binding of serum antibodies was detected with a goat anti-human IgG (Fc)-PE antibody. Subsequently, the Median Fluorescence Intensity (MFI) was assessed on the Luminex MAGPIX® system.

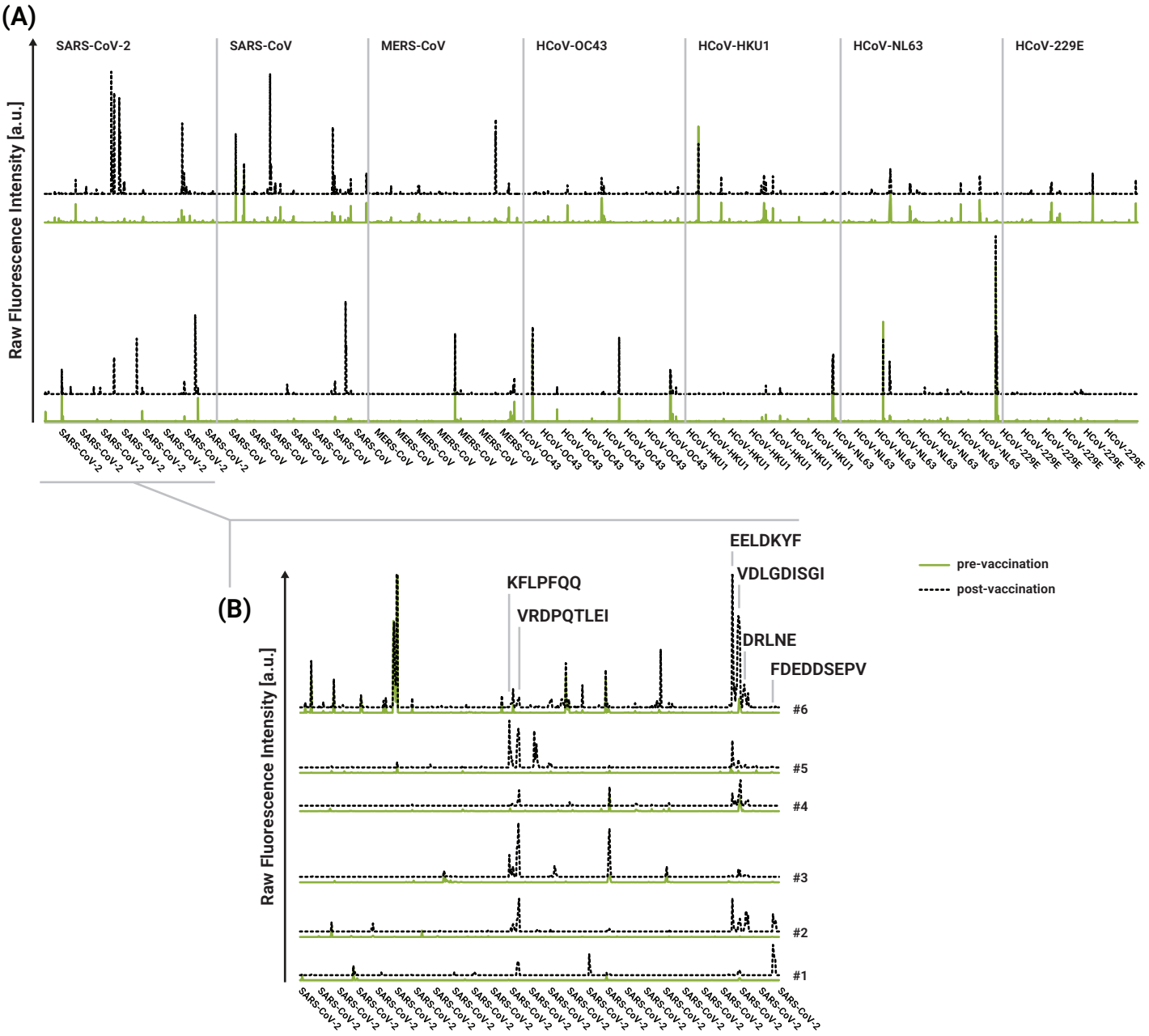

Figure 3. Identification of epitope-specific IgG response pattern against various coronavirus spike proteins after COVID-19 vaccination. PEPperCHIP® Pan-Corona Spike Protein Microarrays were screened with sera collected before and after COVID-19 vaccination (n=6). **(A) IgG reactivity pattern against overlapping spike peptides derived from seven coronaviruses pre- and post- COVID-19 vaccination.** Representative intensity plots showing the IgG-specific epitope pattern pre- (green line) and post- (black, dotted line) vaccination of two vaccine recipients. Raw fluorescence intensity units (y-axis) were plotted against overlapping peptides derived from spike proteins of seven coronaviruses from the N- to the C-terminus (x-axis). **(B) Epitope mapping of IgG responses against SARS-CoV-2 spike protein after vaccination.** Epitopes were annotated when an IgG response in at least two vaccine recipients was detected. Green line = response pattern before vaccination; black dotted line = response pattern after the 2nd dose of COVID-19 vaccination.

Table 1. Peptides used for the bead-based multiplex immunoassay.

ID	Peptide sequence	MagPlex®-Avidin Microspheres bead regions
SARS-CoV-2 Spike E554-D568	ESN**KKFLPFQQ**FGRD-eBio	012
SARS-CoV-2 Spike E574-T588	DAV**RDPQTLEI**LDIT-eBio	015
SARS-CoV-2 Spike Q1142-F1156	QPELDSFK**EELDKYF**-eBio	025
SARS-CoV-2 Spike P1162-V1176	PD**VDLGDISGI**NASV-eBio	029
SARS-CoV-2 Spike N1178-N1192	NIQKEI**DRLNE**VAKN-eBio	033
SARS-CoV-2 Spike C1250-V1264	CGSCCK**FDEDDSEPV**-eBio	036
Human Poliovirus 3 Polyprotein K615-T628	KEVPALTAVETGAT-GG-eBio	020

Bold letters in the peptide sequence highlight the identified epitope (Figure 3B); eBio = Peptides were synthesized with a C-terminal biotin ethylene glycol linker unit.

For the majority of the SARS-CoV-2 spike peptides, IgG responses measured by the bead-based immunoassay were in excellent agreement with the peptide microarray data (Figure 4, Table 2). Only for peptide P1162-V1176, IgG responses could only be partially confirmed.

Considerable strong antibody reactivities were confirmed for SARS-CoV-2 spike peptides E554-D568, D574-T588, Q1142-F1156 and N1178-N1192 by the Luminex MAGPIX® assay (Figure 4B). In accordance with the peptide microarray analysis, peptide C1250-V1264 was also not observed in most of the subjects from the present study with the bead-based system (Figure 3B, Figure 4).

Table 2 shows a side-by-side comparison of the detected IgG responses by peptide microarray analysis and bead-based immunoassay, and at the same time a more detailed view on the individual IgG responses against the identified SARS-CoV-2 peptides as determined by both technologies. An antibody response was considered as positive when signal intensities in post-vaccination sera were twofold higher than the signal in pre-vaccination sera (Table 2). Comparing both technologies, the IgG response pattern against peptides E554-D568, D574-T588, Q1142-F1156, N1178-N1192 and C1250-V1264 matched 80-100% for most of the individuals. In few cases, the vaccine-induced IgG responses analyzed with the Luminex MAGPIX® system did not match with the peptide microarray screening and vice versa.

Overall, exploring the epitope specificity of IgG responses applying peptide microarray screening followed by a multiplexed bead-based immunoassay (Luminex MAGPIX® system) for epitope/peptide validation resulted in a high hit confirmation rate and allows for a straightforward platform transfer of PEPperCHIP® Peptide Microarray data.

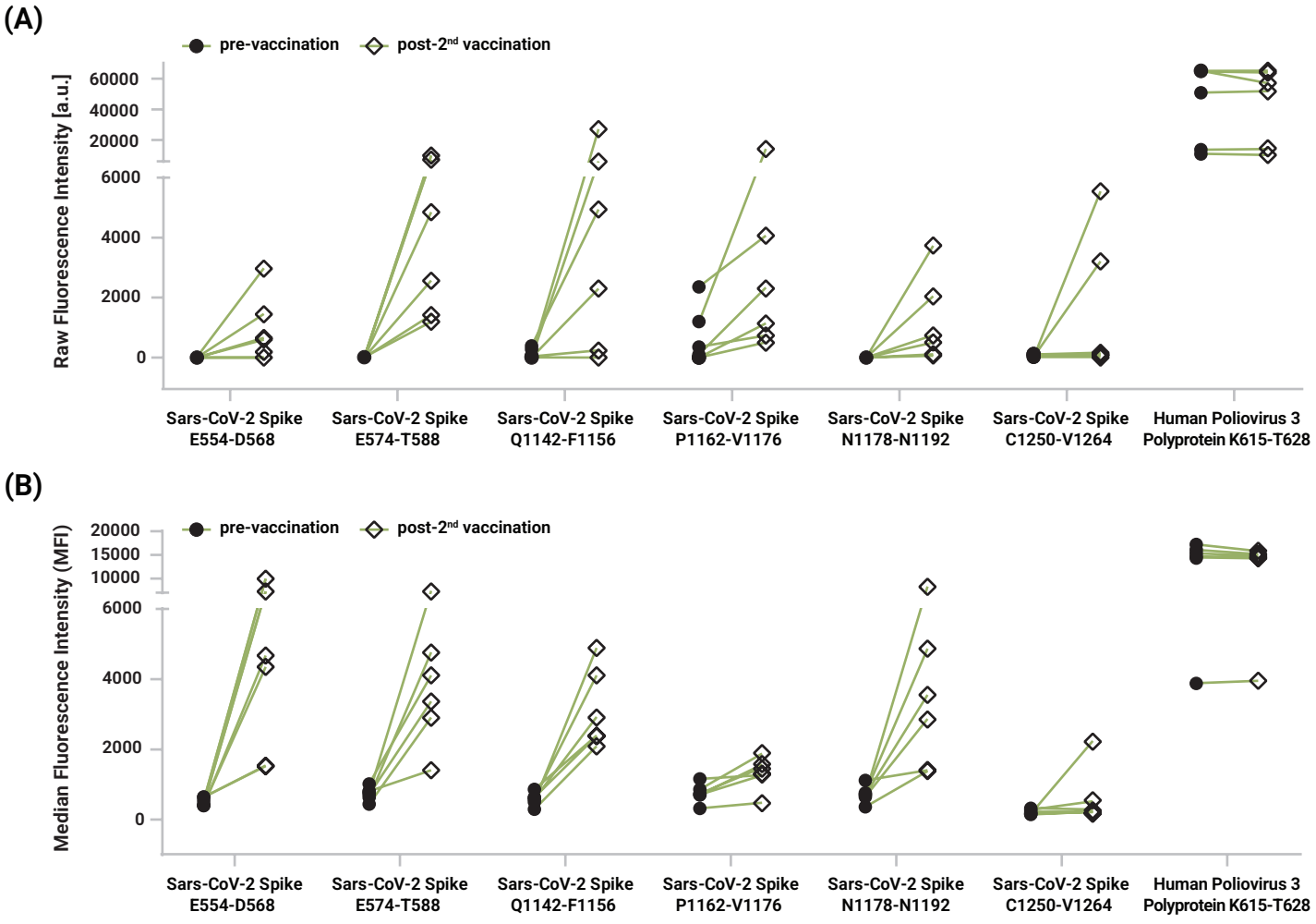

Figure 4. Validation of IgG responses against SARS-CoV-2 spike protein-derived peptides after COVID-19 vaccination. The peptide microarray screening resulted in the identification of IgG-specific SARS-CoV-2 spike protein epitopes, which were further re-evaluated with a multiplexed bead-based immunoassay (xMAP® technology, Luminex). **(A) Peptide microarray data of IgG responses against identified SARS-CoV-2 peptides.** PEPperCHIP® Pan-Corona Spike Protein Microarrays were screened with sera collected pre and post COVID-19 vaccination (n=6). Epitope mapping resulted in six immunogenic peptides showing IgG responses in at least two vaccine recipients. Raw Fluorescence Intensity units (y-axis) were plotted against the identified peptides (x-axis). Shown are individual responses before (black circles) and after vaccination (white squares). IgG responses against a poliovirus epitope served as control. **(B) IgG responses against the same SARS-CoV-2 peptides as analyzed with the Luminex MAGPIX® system.** For each bead set, 5 µg of biotinylated peptide was coupled to 10^6 MagPlex®-Avidin Microspheres. Peptide-coupled microspheres were combined and incubated with sera, serum IgG binding was detected with a PE-conjugated anti-human IgG antibody. The Median Fluorescence Intensity (y-axis) was plotted against the identified peptides (x-axis). Shown are individual responses before (black circles) and after vaccination (white squares). IgG responses against a poliovirus epitope served again as control.

Table 2. Side-by-side comparison of IgG responses in post-vaccination sera as determined by peptide microarray screening and bead-based immunoassay.

	SARS-CoV-2 Spike E554-D568		SARS-CoV-2 Spike E574-T588		SARS-CoV-2 Spike Q1142-F1156		SARS-CoV-2 Spike P1162-V1176		SARS-CoV-2 Spike N1178-N1192		SARS-CoV-2 Spike C1250-V1264	
	A	B	A	B	A	B	A	B	A	B	A	B
#1	-	+	+	+	-	+	+	-	-	-	+	-
#2	+	+	+	+	+	+	+	-	+	+	+	+
#3	+	+	+	+	-	+	+	+	-	+	-	-
#4	-	+	+	+	+	+	-	+	+	+	-	-
#5	+	+	+	+	+	+	+	-	+	+	-	-
#6	+	+	+	-	+	+	+	+	+	+	-	-

Antibody responses were considered as positive when signal intensities in the post-vaccination serum were twofold higher than the signal in the corresponding pre-vaccination serum. A = Peptide microarray data; B = Luminex MAGPIX® data; #1-6 = vaccine-recipient #1-6; (+) = positive IgG response in post-vaccination sera; (-) = no response in post-vaccination sera.

CONCLUSION

This application note presents a workflow using our proprietary peptide microarray technology for peptide/epitope discovery combined with validation by a multiplexed bead-based assay as customary platform for diagnostic tests in a high-throughput format. We applied this approach to identify targets of humoral responses to COVID-19 vaccination and determined immunogenic linear SARS-CoV-2 epitopes of which two were linked to neutralizing IgG responses.

A detailed understanding of antibody responses is of utmost importance not only in the context of vaccination, but also in infection and in other diseases. The described workflow combining the power of large screening capacities for epitope discovery and target validation on a diagnostic platform is ideally suited to identify novel epitope biomarker candidates, which may form the basis of future intervention strategies and diagnostics.

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