

PEPperCHIP® Immunoassay Protocol

Version 09/17

To ensure optimal assay conditions and best data quality,
 please follow the immunoassay protocol with your antibody or serum samples
 for high-resolution epitope mapping, serum biomarker discovery or similar experiments.

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1. What you receive from PEPperPRINT

1.1 Custom PEPperCHIP® or Standard Peptide Microarrays and storage

[PEPperCHIP® Peptide Microarrays](#) are provided on standard glass chips (3" x 1", 75.4mm x 25.0mm x 1.0 mm). We provide 1 to 8 and 16 subarray copies per chip. Multiple array copies can be assayed separately with a suited PEPperCHIP® Incubation Tray. The chips should be stored at 4°C, in the dark under inert gas atmosphere to ensure long shelf life. If handled with care chips are stable for months. For chip handling see page 4.

1.2 PEPperCHIP® Incubation Tray

The [PEPperCHIP® Incubation Tray](#) allows working with minimal sample volumes and staining of three chips in parallel. It also enables one-by-one assays with multiple subarray copies per chip. The incubation trays are available in subarray formats of 1 to 8 and 16 subarray copies per chip and are thus compatible with all available chip layouts. PEPperCHIP® Incubation Trays are fully reusable with minimal sample consumption in line with immunoassays. The handling of the incubation tray is described on page 4.

1.3 PEPperCHIP® anti-HA control antibody

PEPperCHIP® Peptide Microarrays contain influenza virus antigen hemagglutinin (HA) epitopes as [standard positive controls](#), usually arranged as single spots as a frame around the main content or for content segmentation. We provide monoclonal mouse anti-HA DyLight680 (12CA5) control antibody with excitation / emission wavelengths of 692 nm / 712 nm suitable for multiple microarray scanners (see page 3). We recommend a working dilution of 1:2,000. The antibodies should be stored at 4°C or -20°C for long time storage.

1.4 PepSlide® Analyzer software

[PepSlide® Analyzer](#) is an intuitive and easy-to-handle software solution for peptide microarray image analysis and supports quantification of microarray data, the interpretation of peptide content and spot morphology as well as serves as a statistical data mining tool. The software links microarray design files (*.psf*) with microarray content information (*.tiff*) for analysis. The manual of the PepSlide® Analyzer is available in the [download section](#). If ordered, you receive the software license key with your microarray data package.

2. Additional equipment and buffer that you need

2.1 Equipment

- **Orbital shaking device**

- **Microarray scanner**

PEPperCHIP® Peptide Microarrays are compatible with most microarray scanners, e.g.:

- GenePix® 4000 A/B, 4100B, 4200L and 4300/4400 microarray scanners
- Tecan LS Reloaded™ and PowerScanner™ microarray scanner
- Agilent High-Resolution and SureScan microarray scanner
- Innopsys InnoScan® 700, 710 and 900 microarray scanner
- NimbleGen MS 200 microarray scanner
- LI-COR Odyssey® imaging system scanners
- many more e.g. Eppendorf, GE Healthcare, PerkinElmer, etc.

The scanner should be compatible with 75mm x 25mm microscope slides and read fluorescent intensities from **DyLight680, DyLight800 or Cy3 and Cy5 labels** with a **resolution of 20 µm** or higher.

- **Secondary antibody with fluorescent label**

The fluorescently labelled secondary antibody is needed to stain your primary sample (e.g. serum or antibody). Please note that strongly charged fluorescent dyes can interact with acidic and basic side chains of peptides. Therefore, we recommend the use of neutral dyes or dyes with few charged functional groups (e.g. DyLight680, DyLight800, Cy3 or Cy5), as well as pre-incubation with secondary antibodies to screen for background interactions.

- **Your primary sample** (e.g. antibody, serum, or other)

2.2 Buffer

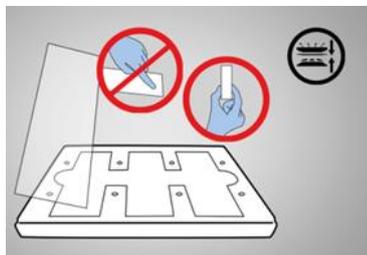
- **Standard buffer:** Phosphate-buffered saline (PBS) with 0.05% Tween20, pH 7.4
- **Blocking buffer:** Rockland Blocking Buffer MB-070 or standard buffer with 1% BSA
- **Staining buffer:** PBS with 0.05% Tween20 and 10% blocking buffer
- **Dipping buffer:** 1 mM Tris, pH 7.4

Please note:

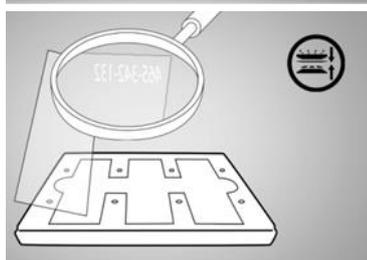
The blocking buffer may affect the signal intensities of your peptide microarray. Highly efficient blocking buffers like BSA or milk powder can reduce the intensity of a given interaction by a factor of 10 or higher.

3. Incubation tray and chip handling

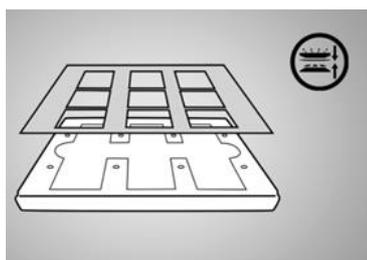
This section summarizes the handling of PEPperCHIP® Peptide Microarrays and PEPperCHIP® Incubation Trays. We also recommend watching the [short tutorial movie](#).



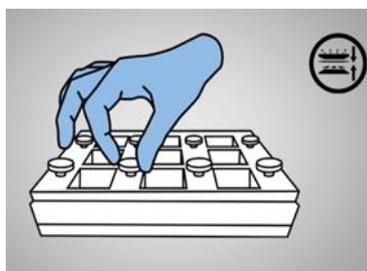
- PEPperCHIP® Microarrays should be handled with care and should be touched only at the edges of the chip.
- Do not touch the chip surface at any time! Avoid dust or other microscopic contamination and wear non-powdered laboratory gloves.



- An individual chip number on the back side marks each chip.
- The chip is **correctly placed** in the cavities of the base plate when the **mirrored chip number appears in the upper right corner**. Fill empty chip cavities with dummy chips.



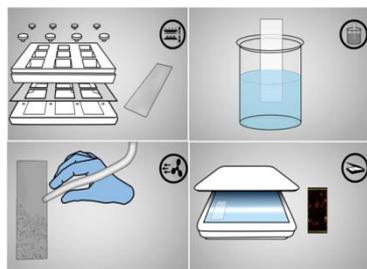
- Place the seal with the **glossy side facing downwards**.
- The **holes of the seal should be placed centrally** onto the corresponding holes of the base plate.
- **Do not move or lift the seal once it is placed!**



- Assemble the incubation tray.
- Tighten thumbscrews one after another in a diagonal manner.
- **Do not fasten with pressure!**



- **Slowly add solutions or aspirate liquid** into / from the array chamber corners without touching the peptide microarray surface.
- Tilt the incubation tray if needed.
- **Cover** the peptide microarrays **with the lid** to avoid liquid evaporation or bleaching of fluorophores during incubation steps.



- **Disassemble the incubation tray** before scanning.
- **Dip the chip** into dipping buffer 2 to 3 times and **dry the chip in an air stream** blowing from top to bottom **until it is completely dry**.
- **Place the chip onto a microarray scanner with the mirrored chip number in the upper right corner.**
- **Clean the incubation tray with mild detergents (e.g. 2% Microbac® forte) for reuse.**

4. Immunoassay protocol

4.1 Recommended incubation volumes

The PEPperCHIP® Incubation Tray allows working with minimal liquid volumes for optimal wetting of the chip. In the following table, minimal incubation volumes are listed for each of the different array formats:

PEPperCHIP® subarray format	Minimal incubation volume per array
1 array per chip	1,500 µl
2 arrays per chip	700 µl
3 arrays per chip	400 µl
4 arrays per chip	350 µl
5 arrays per chip	200 µl
16 arrays per chip	100 µl

4.2 Recommended sample and antibody dilutions

In the following table the recommended starting dilutions and concentrations for each sample type are listed. **Please be aware that dilutions may vary depending on nature of the sample and interaction parameters.** Low antibody titers in sera, high off-rates and weaker affinities of mono- and polyclonal antibodies require higher sample concentrations. Higher concentrations of secondary antibody can cause stronger background signals.

Your sample type	Range	Recommended starting dilution or concentration
Purified antibody	1 - 500 µg/ml	1 µg/ml
Serum: IgG, IgA, etc.	1:10 - 1:10,000	1:1,000
Serum: IgE	1:2 - 1:1,000	1:5
Cerebrospinal fluid (CSF)	1:10 - 1:10,000	1:100
Secondary antibody	1:500 - 1:10,000	1:5,000
Anti-HA control antibody	1:2,000	1:2,000

4.3 Important notes before you start

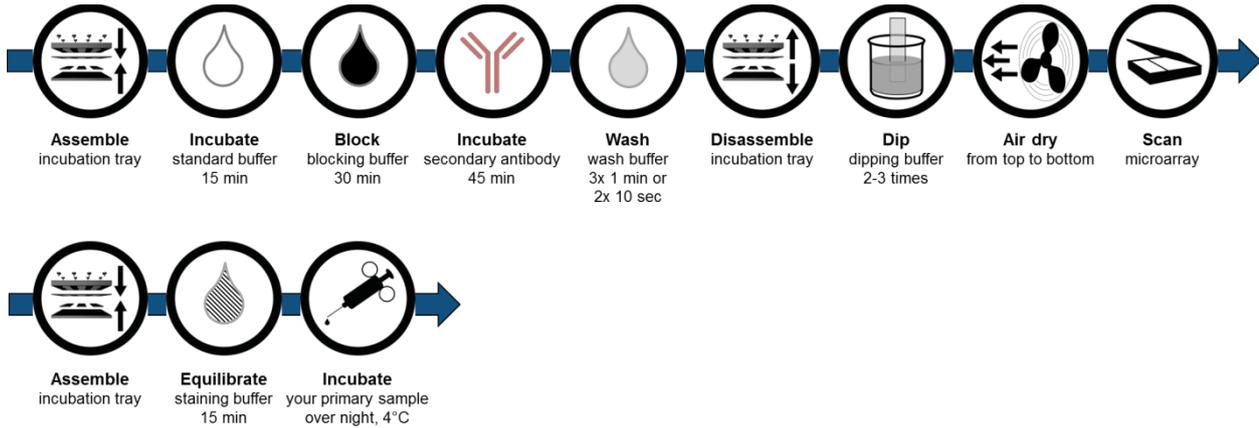
- PEPperCHIP® Peptide Microarrays should be handled with care. **Only touch the peptide microarray chips at the rims with your fingers or use forceps with a wide tip in the area where the chip number is engraved.**
- Place the chip into the cavities of the incubation tray with the **mirrored chip number appearing in the upper right corner.**
- **Place the seal of the incubation tray at the exact intended position during assembly steps.** During incubation tray disassembly, the seal must be removed carefully without touching the chip surface.
- Do not add solutions directly onto the array area. Instead, **pipette with the pipette placed close against the incubation tray wall.** Aspirate liquid from the array chamber corners by slightly tilting the incubation tray.
- Aspirate and add solutions quickly. **Do not let the microarray surface dry** during the immunoassay procedure. Completely dry the peptide microarray just moments before scanning.
- We recommend using an **orbital shaker at 140 rpm** during incubation steps for optimal wetting. This will improve sample circulation and avoid liquid gradients, which may cause de-wetting and bias in microarray data.
- Vigorous washing may lead to the loss of sample from the peptide microarray when incubated with low affinity and/or high off-rate samples e.g. **conformational epitopes.** If necessary, reduce **washing time to 2 x 10 seconds.**
- Evaporation of liquids and bleaching of antibody fluorophores during incubation steps can be prevented by **placing the lid on the incubation tray.**
- If **signal intensities and/or signal-to-noise ratios are too low**, the same subarray can be incubated again using your sample with a lower serum dilution or higher antibody concentration. Repeat incubation with your primary sample and staining with secondary antibody (continue with step 10).
- To shorten experimental time, staining with fluorescently labeled secondary antibody can be combined with anti-HA control antibody in one step if your primary sample is NOT originating from mouse (e.g. mouse antibody or serum) and if both antibodies are labelled with different fluorophores.

4.4 Immunoassay quick start guide

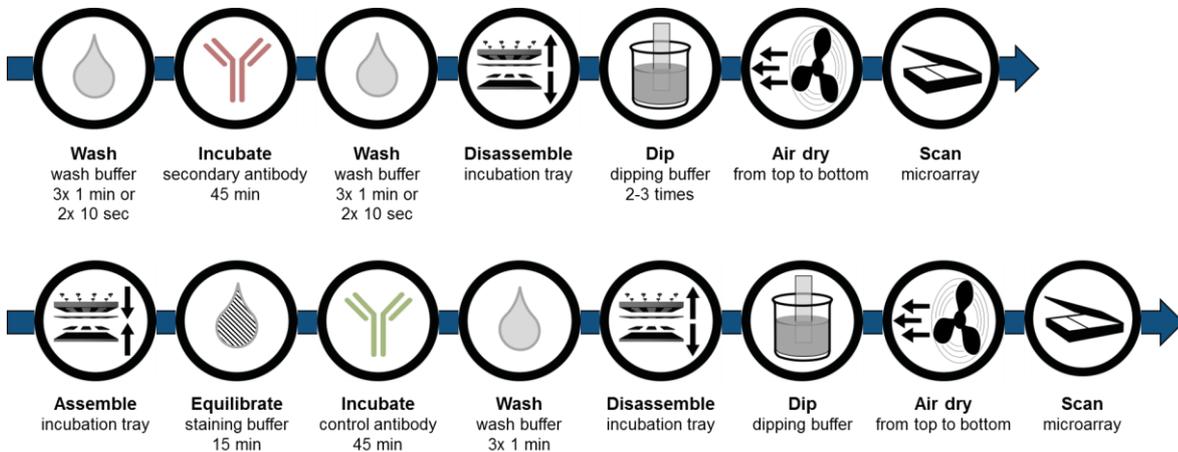
This section illustrates the two-day experimental workflow of immunostaining your PEPperCHIP® Peptide Microarray, which is also described in detail on pages 8 to 10.

Steps necessary to be followed for fast and easy immunostaining are:

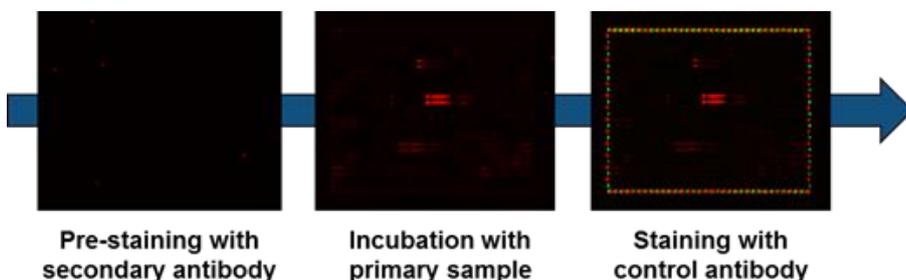
Day 1: Pre-staining with secondary antibody and incubation with your primary sample (approximate total experimental time: 2.5 to 3 hours):



Day 2: Staining with secondary antibody and anti-HA control antibody (approximate total experimental time: 2.5 to 3 hours):



Depending on your peptide microarray content your scans should have a similar appearance as depicted below:

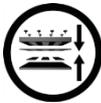


4.5 Pre-staining with secondary antibody

Secondary antibody used for staining of your primary sample may interact with the peptides printed on the chip. To discriminate such background interactions from sample-specific signals, we recommend a pre-staining with secondary antibody and pre-analysis of the PEPperCHIP® Peptide Microarray. This can be done either on the subarray used for incubation of your sample or any other array copy, if available. Background signals can be subtracted from signals obtained from your sample during data analysis.

-  Before assembling the PEPperCHIP® Incubation Tray, bring the peptide microarray to room temperature to avoid formation of water condensation. Afterwards, assemble the PEPperCHIP® Incubation Tray as described on page 4.
-  Incubate the peptide microarray for 15 min at room temperature in standard buffer (PBS, 0.05% Tween20, pH 7.4) using an orbital shaker at 140 rpm for optimal wetting. Check carefully for incubation tray leakiness and tighten the thumbscrews if necessary. Aspirate the buffer afterwards.
-  Block the peptide microarray for 30 min at room temperature with blocking buffer (Rockland blocking buffer or standard buffer with 1% BSA). Use an orbital shaker at 140 rpm. Aspirate the buffer afterwards.
-  Stain with secondary antibody diluted in staining buffer (PBS, 0.05% Tween20, 10% blocking buffer) for 45 min at room temperature using an orbital shaker at 140 rpm, protected from light. Dilute the secondary antibody as described on page 5 in chapter 4.2. Completely aspirate the antibody solution.
-  Wash the peptide microarray for 3x 1 min with standard buffer using an orbital shaker at 140 rpm, protected from light (wash 2x 10 sec when using conformational peptide microarrays). Completely aspirate the buffer afterwards.
-  Disassemble the PEPperCHIP® Incubation Tray by opening all thumbscrews. Carefully take out the peptide microarray chip.
-  Directly dip the chip 2 to 3 times into dipping buffer filled in a beaker. If necessary, tilt the beaker. Repeat this step until all visible contamination (e.g. PBS residues or dust) is removed.
-  Carefully dry the PEPperCHIP® Peptide Microarray completely in a stream of air from top to bottom.
-  Scan the peptide microarray according to guidelines of your scanner using the appropriate fluorescence channels. The chip is correctly placed onto the scanner when the chip number appears in the upper right corner in mirror view. Export the images for each channel and save them as a 16-bit grayscale (.tiff) image.

4.6 Incubation with your primary sample

10.  Re-assemble the incubation tray as described on page 4.
11.  Equilibrate the PEPperCHIP® Peptide Microarray in staining buffer (PBS, 0.05% Tween20, 10% blocking buffer) for 15 min at room temperature on an orbital shaker at 140 rpm. Check carefully for incubation tray leakiness and tighten the thumbscrews if necessary. Aspirate the buffer afterwards.
12.  Incubate your primary sample diluted in staining buffer overnight at 2 to 8 °C on an orbital shaker at 140 rpm. Dilute your primary sample as recommended and described on page 5 in chapter 4.2.
13.  Wash the peptide microarray for 3x 1 min with standard buffer (PBS, 0.05% Tween20, pH 7.4) using an orbital shaker at 140 rpm (wash 2x 10 sec when using conformational peptide microarrays). Completely aspirate the buffer. Due to short washing periods, prepare the secondary antibody solution for step 14 beforehand.
14.  Stain with secondary antibody diluted in staining buffer (PBS, 0.05% Tween20, 10% blocking buffer) for 45 min at room temperature using an orbital shaker at 140 rpm, protected from light. Dilute the secondary antibody as described on page 5 in chapter 4.2. Completely aspirate the antibody solution.
15.  Wash the peptide microarray for 3x 1 min with standard buffer using an orbital shaker at 140 rpm (wash 2x 10 sec when using conformational peptide microarrays) protected from light. Completely aspirate the buffer afterwards.
16.  Disassemble the PEPperCHIP® Incubation Tray by opening all thumbscrews. Carefully take out the peptide microarray chip.
17.  Directly dip the chip 2 to 3 times into dipping buffer filled in a beaker. If necessary, tilt the beaker. Repeat this step until all visible contaminations (e.g. PBS residues or dust) are removed.
18.  Carefully dry the PEPperCHIP® Peptide Microarray completely in a stream of air from top to bottom.
19.  Scan the peptide microarray by placing it onto the scanner. The chip number should appear in the upper right corner in mirror view. Export the images for each channel and save them as a 16-bit grayscale (.tiff) image. If signal intensity and/or signal-to-noise ratio is too low, repeat steps 10 to 19 with increased sample concentration.

4.7 Staining with anti-HA control antibody

20.  Re-assemble the incubation tray as described on page 4.
21.  Equilibrate the PEPperCHIP® Peptide Microarray in staining buffer (PBS, 0.05% Tween20, 10% blocking buffer) for 15 min at room temperature on an orbital shaker at 140 rpm. Check carefully for incubation tray leakiness and tighten the thumbscrews if necessary. Aspirate the buffer afterwards.
22.  Incubate with PEPperCHIP® anti-HA control antibody diluted in staining buffer for 45 min at room temperature protected from light. We recommend a working dilution of 1:2,000. Use an orbital shaker at 140 rpm. Aspirate the buffer afterwards.
23.  Wash the peptide microarray for 3x 1 min with standard buffer (PBS, 0.05% Tween20, pH 7.4) using an orbital shaker at 140 rpm, protected from light (wash 2x 10 sec when using conformational peptide microarrays). Aspirate the buffer afterwards.
24.  Disassemble the PEPperCHIP® Incubation Tray by opening all thumbscrews. Carefully take out the peptide microarray chip.
25.  Directly dip the chip 2 to 3 times into dipping buffer filled in a beaker. If necessary, tilt the beaker. Repeat this step until all visible contaminations (e.g. PBS residues or dust) are removed.
26.  Carefully dry the PEPperCHIP® Peptide Microarray completely in a stream of air from top to bottom.
27.  Finally, scan the peptide microarray by placing it onto the scanner. The chip number should appear in the upper right corner in mirror view. Export the images for each channel and save them as a 16-bit grayscale (.tiff) image. Continue with data analysis using the PepSlide® Analyzer software.

5. Data analysis with the PepSlide® Analyzer

PEPperCHIP® Peptide Microarrays are provided with an electronic data package including the microarray design file (.psf) that assigns the peptide sequences to the spots, the Excel spreadsheet with your peptide map as well as the PepSlide® Analyzer license key.

You can download the PEPSlide® Analyzer for [32-bit Windows](#), [64-bit Windows](#) and [Mac OS X](#). After installation, activate the software with the PepSlide® Analyzer license key.

In the following section, you can find a quick guideline on how to analyze peptide microarray data. It is also described in detail in the [PepSlide® Analyzer Quick Start Guide](#) and [PepSlide® Analyzer User Guide](#).

1. Data input

- Open the PepSlide® Analyzer software and enter the license key.
- Choose *Images > Open Image* and select the scanned images in (.tiff)-formats of all acquired fluorescent channels from step 27 on page 10.
- Assign signals to the red or green channel depending on the fluorescent labels.

2. Image processing

- Assure that your image is in the correct orientation. If necessary, use the *Zoom In* and *Zoom Out* options, rotate or flip the image vertically or horizontally to obtain an appropriate image view.

3. Load array file

- Choose *Arrays > Open Array* and select the appropriate (.psf) file.
- Please note: all spots are grouped in spot families visible as a grid according to the peptide microarray layout design and peptide content.

4. Array alignment

- Select the spot family of interest or select the whole grid by pressing *Ctrl-A*. Drag and drop the grid until spots and grid overlay.
- If necessary, chose *Image* and change the contrast for better image visualization.
- If the spots do not exactly correspond to the array grid, use the *Array Rotation* function.

5. Data quantification

- Choose *Quantify* in the control panel and choose the quantification setting according to your needs.
- Use *Quantify selection* to calculate spot signal intensities

6. Data export

- Choose *Export to CSV* to save all necessary data.
- Use Excel or other calculation software for further statistical analysis and result visualization.

6. Troubleshooting

There are scratches or blurry areas on my scan.

- The chip surface is very vulnerable to contact. Any sharp instrument will damage the microarray surface and thereby make analysis difficult.

Solution: Do not touch the microarray surface at all. Hold the chip only with forceps with a wide tip in the area of the chip number.

There are artifacts on my chip and they cover my signals.

- The seal has been placed and removed multiple times during incubation tray assembly and disassembly.

Solution 1: Place the seal very precisely onto the chip, and do not move it anymore after it has touched the chip surface.

- The liquid on the chip has dried out.

Solution 2: To prevent such artifacts, directly dip the chip after washing steps and dry it immediately using a stream of air. If you are using the PEPperCHIP® Peptide Microarrays for the first time, it is advisable to stain only three chips at once

Solution 3: Avoid rocking incubation and rather use an orbital shaker at 140 rpm.

Signal intensities are weak and/or signal-to-noise ratios are low.

- One reason can be the blocking buffer. Highly efficient blocking buffers containing BSA or milk powder can reduce the intensity of a given interaction by a factor of 10 or higher.

Solution 1: We recommend Rockland Blocking Buffer MB-070 or standard buffer with 1% BSA.

- Vigorous washing may be another reason.

Solution 2: For low affinity interactions and/or high off-rates, we recommend reducing washing times.

Solution 3: For conformation arrays, washing can be reduced down to 2x10 sec,

- The same array can also be stained again with a lower serum dilution or higher antibody concentration of your primary sample.

Solution 4: Repeat staining with your primary sample and secondary antibody.

The background signal is too high.

- Strongly charged fluorescent dyes can interact with acidic and basic side chains of peptides.

Solution: We recommend the use of neutral dyes or dyes with few charged functional groups (e.g. DyLight680, DyLight800, Cy3, Cy5), as well as pre-incubation with secondary antibodies to screen for background interactions.

7. Contact details for technical support

For further questions and technical support, please do not hesitate to contact us (details below) or your local sales representative (details on our website):

Phone: +49 6221 7264489
 Fax: +49 6221 7264475
 Email: pepperchip@pepperprint.com

Website: www.pepperprint.com
 LinkedIn: <https://de.linkedin.com/company/pepperprint-gmbh>
 Twitter: <https://twitter.com/pepperchip>

Address: PEPperPRINT GmbH, Rischerstrasse 12, 69123 Heidelberg, Germany

