Protocol

RepliTope™ Antigen Collection Microarrays

Ready-to-use peptide microarrays for antibody profiling

Revision 3.2

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Table of content

1 Introduction

Antibody-antigen interactions are key events in immunology. Therefore, the identification of epitopes or immunodominant regions in antigens represents an important step in the characterization of antibodies. One of the most efficient ways to identify such epitopes is incubation of a collection of antigen-derived peptides displayed on glass slides (RepliTope™ peptide microarrays) with antibodies of interest.

JPT Peptide Technologies' RepliTope™ antigen collection peptide microarrays represent catalog peptide microarray slides for rapid screening of protein-peptide interactions. The purified peptides displayed on glass slides are chemoselectively and covalently bound, enabling effective interaction with binding partners. Immobilized sequences represent overlapping peptides derived from multiple antigens allowing efficient profiling of humoral immune responses using patient samples and protein-protein interaction studies. In addition, full proteome RepliTopes™ are available that cover overlapping peptide scans through complete microbial or viral proteomes. Upon incubation with your protein or patient sample the binding event can be detected by reading out photon intensity emanating from the fluorescently labeled primary or secondary antibody.

2 List of Components

1. **RepliTope™ peptide microarray**

   Glass slide displaying peptides in three identical subarrays (see: 4.2)

2. **Blank slides engraved with *"Dummy"* or *"Blank"***

   One blank slide per PepStar™ Peptide Microarray

3. **JPT Peptide Microarray Spacers**

   Vials containing 20 spacers each

   Two spacers per PepStar™ peptide microarray are needed

4. **Product Documantation**

   Relevant files for the specific peptide microarray (protocol and datasheet as pdf-files, sequence info as gal-file and JPT's GalViewer software as zipped package)

3 Storage and Handling

3.1 Storage of RepliTope™ Peptide Microarray Slides

- Optimal storage conditions for peptide microarray slides are in a cool (approx. 4°C / 39°F) and dry environment.
- Peptide microarrays are stable for at least 6 months when stored at 4°C (39°F).
- Do **not** freeze the peptide microarrays for prolonged storage.

3.2 Handling of RepliTope™ Peptide Microarray Slides

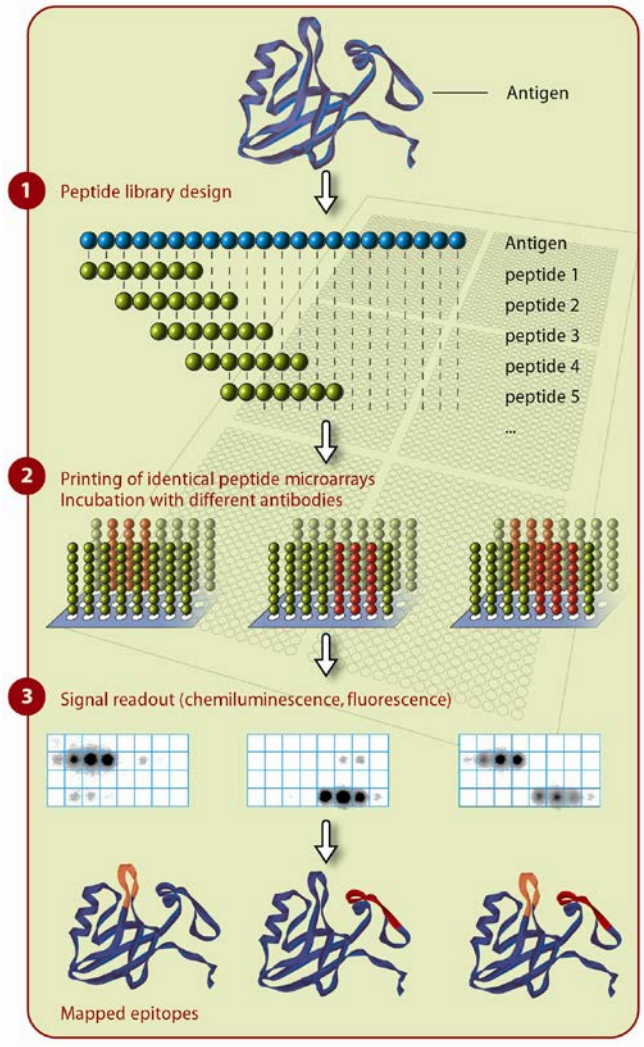
- Always handle the delicate peptide microarrays with care.
- Never touch the peptide microarray slide surface.
- Always wear laboratory gloves when handling peptide microarray slides.
- Please hold peptide microarray slides at the end, which carries the engraved data label. This label provides a unique identification of the specific microarray.
- Please take care when dispensing solutions onto the slide surface. Make sure not to touch the surface with pipette-tips or dispensers.
- Never whisk the surface of the peptide microarray slide with a cloth.
- Never use chemicals other than described. Inappropriate chemicals may destroy the chemical bonding of the peptides to the glass surface.
- Avoid dust or other particles during each step of the experiment. Dust, particles and resulting scratches will cause artifacts during final signal readout.
- Please filter all solutions for the washing steps with minimum 2µm, preferably 0.4µm, particle filters before use.

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING YOUR EXPERIMENTS! CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF REPLITOPE™ PEPTIDE MICROARRAYS.
PLEASE CONTACT JPT PEPTIDE TECHNOLOGIES´ TECHNICAL SERVICE FOR ASSISTANCE OR FOR QUESTIONS.

4 General Considerations

4.1 Experimental Basics

JPT Peptide Technologies' RepliTope™ antigen collection peptide microarrays comprise purified synthetic peptides, derived from antigens (principle of epitope detection see Figure 1) or other sources, that are chemoselectively and covalently immobilized to the glass surface. An optimized hydrophilic linker moiety is inserted between the glass surface and the antigen derived peptide sequence to avoid false negatives caused by sterical hindrance. For technical reasons all peptides contain a C-terminal glycine.



JPT's RepliTope™ antigen collection peptide microarrays are designed for detecting potential biomarkers for infectious diseases, autoimmune diseases, cancer and allergies and to elucidate protein-protein interactions. Each spot in the microarray represents a single individual peptide.

After incubation of the peptide microarray with human patient serum, bound antibodies can be detected using fluorescently labeled anti-human-IgG antibodies

Resulting antibody signatures represent unique insights into the individual humoral immune status.

Figure 1: General principle of epitope detection using overlapping peptide scans.

All peptides are displayed in three identical subarrays on each slide. RepliTope™ slide surfaces are delivered in a pre-treated form minimizing unspecific binding of your target protein. Therefore, usually no blocking step is needed.

4.2 RepliTope™ Peptide Microarray Layout

Please refer to the .gal-file provided with the product documentationfor identity and location of the spots on the microarray surface. The microarray side carrying the engraved label represents the surface displaying the attached peptides. The .gal-file can be opened using microarray evaluation software-modules capable of evaluating high-density microarray slides or the GalViewer-software. Since .gal-files are tab-separated text files, they can also be processed with software modules such as Microsoft Editor (Notepad) or Microsoft Excel. A schematic layout of the peptide microarray is shown in Figure 2.

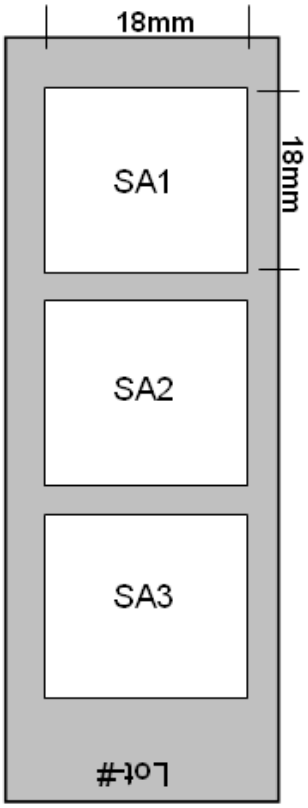


Figure 2: Schematic layout of a peptide microarray (SA=subarray).

As shown in Figure 2 the peptide microarray is printed in three identical subarrays (SA). This enables highly efficient intra-chip-reproducibility tests using scatter plots or correlation functions.

Each peptide is printed one time per subarray. With the .gal-file provided, evaluation can be performed using software modules like GenePix, ArrayPro or similar programs, which align the .gal-file induced grid onto the resulting image. JPT´s GalViewer software can be applied for qualitative analysis and spot identification.

For RepliTope™ antigen collection microarrays, each subarray (SA) is printed in individual blocks (see Figure 3, details in Figure 4). The number of blocks and the final layout will vary according to the final number of peptides derived from the antigens.

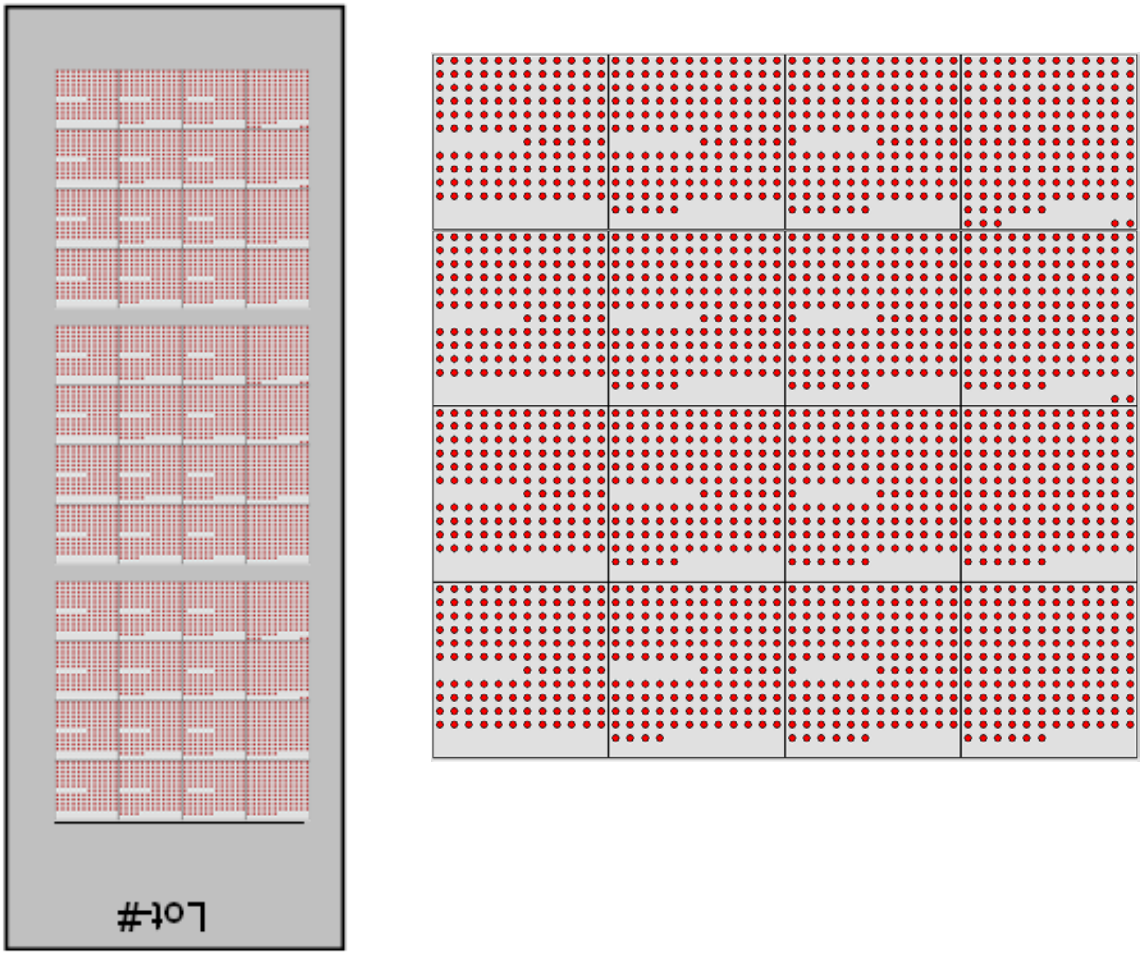


Figure 3: Exemplary view for a microarray slide with 3 subarrays (left) and a subarray consisting of 16 individual blocks (right).

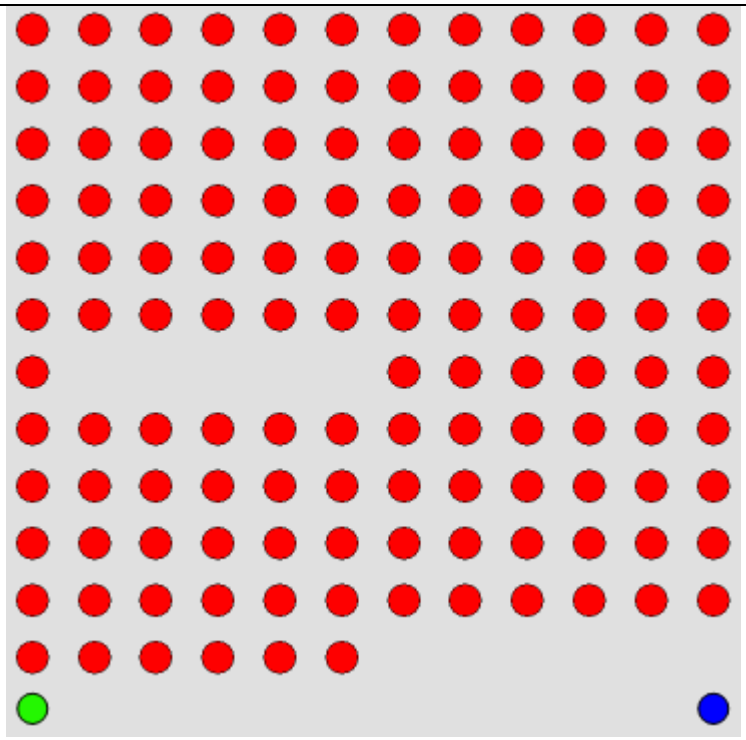


Figure 4: Exemplary block layout

Each spot represents an individual peptide. Green spot refers to process control (flag-tag-peptide), blue spot to fluorescent landmark (printed only in selected blocks, for details please refer to .gal-file). Blank spots between individual antigen scans are devoid of any peptide.

5 Experimental Protocols

Note: The following procedure is given as a guideline only. The optimal experimental conditions will vary depending on the experiment and cannot be predetermined - they must therefore be established by the user. No warranty or guarantee of performance using this procedure with your target protein or serum can be made or is implied.

The RepliTope™ peptide microarray is designed as a ready-to-use product to identify epitopes, peptide binders or immunodominant regions in antigens directly on the glass slide surface.
Ordinarily, there is no need to perform blocking steps on the slide surface prior to incubation with the target sample. However, in case of incubations with patient sera or plasma, JPT recommends to include an additional blocking step prior to incubation with patient sample.
Please refer to the .gal-files provided with the product documentation for identity and location of the spots on the peptide microarray surface. The side of the slide displaying the peptides is marked with the engraved lot number.

Note: For analysis of protein/protein-interaction no specific guideline can be provided. Several factors such as buffer components, ion strength, pH-value, temperature, washing conditions and more may influence the binding affinity of the target protein to the immobilized peptides. JPT also recommends to perform a direct labeling reaction of the protein of interest as well as several independent incubations covering different conditions such as concentrations, temperatures and washing procedures.

5.1 Additional Materials Required

1. **Analyte:**
   a. **Primary antibody**

      JPT recommends a final concentration of about 1-10 µg/ml

   b. **Proteins / enzymes**

      For analysis of e.g. protein binding components, JPT recommends a final concentration of 10 µg/ml or above

   c. **Blood sera or plasma solution**

      Final sample dilution of 1:100 to 1:500 in blocking buffer

2. **Secondary antibody**

   Fluorescently labeled 2nd antibody (Note: JPT recommends DyLight 649 or related far-red-fluorescent dyes and a final concentration of about 1 µg/ml. Blue and green dyes are not recommended due to background issues.)

3. **Optional: Labeling Kit for Proteins / Antibodies**

   For direct labeling of proteins or antibodies JPT recommend to use the following kit: DyLight™ 650 Microscale Antibody Labeling Kit (ThermoFisher Scientific; 84536). Please follow the instruction of use delivered together with the kit for preparing your sample

4. **Blocking buffer**

   For sample dilution (JPT recommends usage of Superblock T20 buffer (Thermo/Pierce, #37516) or alternatives like e.g. 3% BSA in 1x TBS-Buffer + 0.1% Tween20 (TBS-T))

5. **Washing buffer**

   1x TBS-Buffer + 0.1% Tween20 (TBS-T)

6. **De-ionized water**

   For final washing steps of the microarrays

5.2 Additional Hardware and Software

1. **Tweezers**

   For handling of PepStar™ Peptide Microarrays

2. **Automated Incubation/Hybridization Station**

   Tecan Hybridization Station HS4X00

   Note: Alternatively, incubation in a microarray-chip-sandwich format or in a incubation trough can be used. Please refer to point 5.3.2 for further details

3. **4-Well Dish, Microscope Slide Staining Dish or 50 mL-Falcon Tubes (Incubation trough)**

   For manual incubation and washing steps

4. **Rocking Platform**

   For all manual incubation and washing steps (Note: Do not shake Microarray-Chip-Sandwiches!)

5. **Microarray Centrifuge**

   Or access to a stream of nitrogen to dry the microarray slides

6. **Fluorescence Scanner/Imager**

   Capable of excitation of appropriate fluorophore moiety and with a resolution of at least 10 µm per pixel

7. **Analysis Software**

   Allowing quantification of the image and the assignment of signal intensities to individual peptides using the provided gal-file

5.3 Incubation Procedure

5.3.1 Fully Automated Microarray Processing

All peptide microarrays produced by JPT have an identical layout concerning active area and spotted surface. Although the content of the microarrays varies, the overall layout and dimensions are the same (see Figure 5).

	Please check with the manufacturer of your microarray processing station for compatibility with the required liquids. Most microarray processing stations are sensible towards strong acids and organic solutions. Protocols have to be adapted to prevent permanent damage to your device.

All peptide microarrays produced by JPT are adjusted to fit into common fully automated microarray processing systems. JPT recommends using Tecan HS4X00 Hybridisation systems. All RepliTope™ microarrays are printed according to the layout of the Single chamber option of Tecans HS4X00 Pro station.

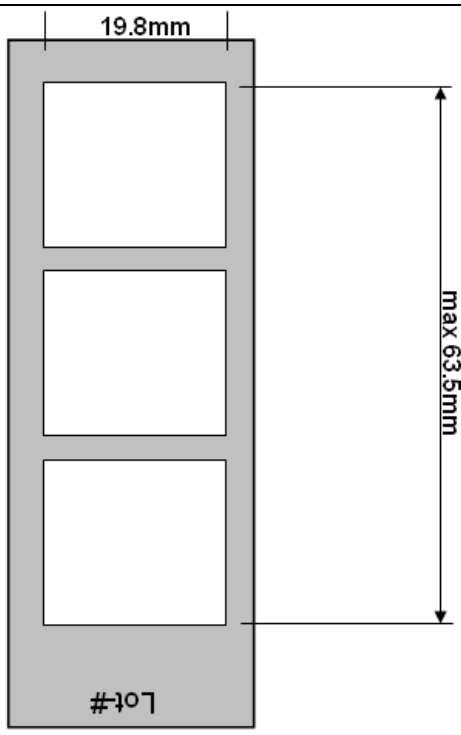


Figure 5: Maximum area dimension on JPT peptide microarrays.

An exemplary protocol for the use of JPT's RepliTope™ peptide microarrays in Tecan's HS4X00 processing machine is shown Figure 6.

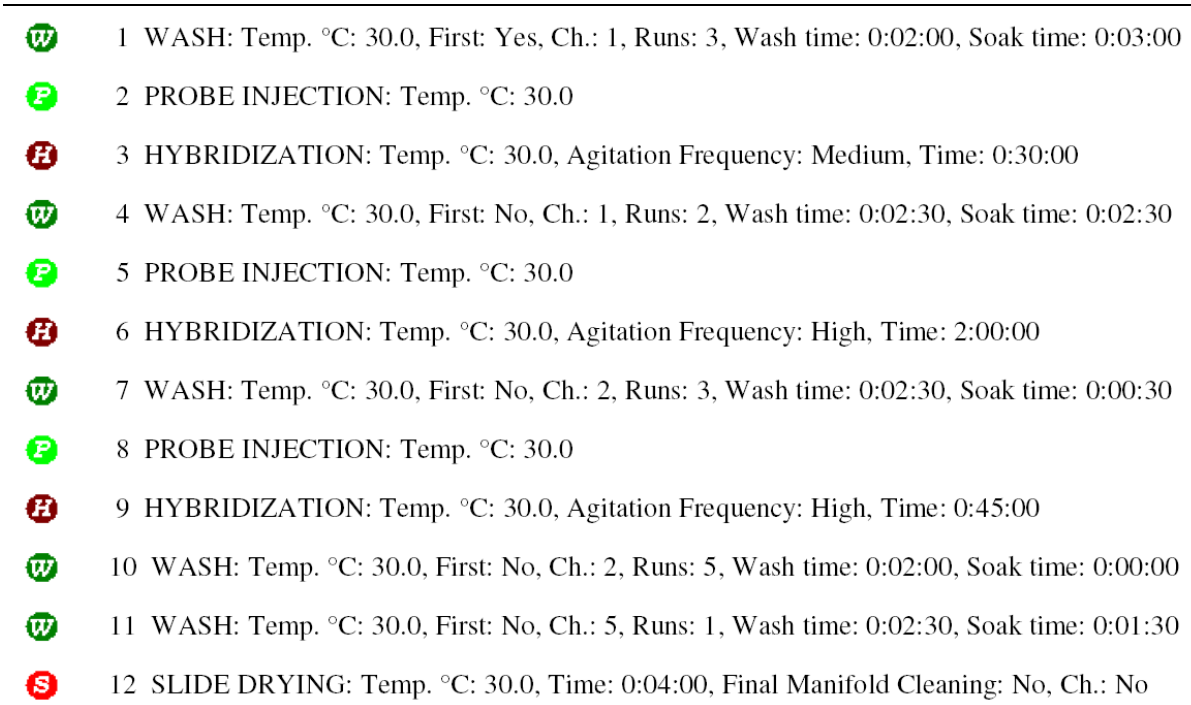
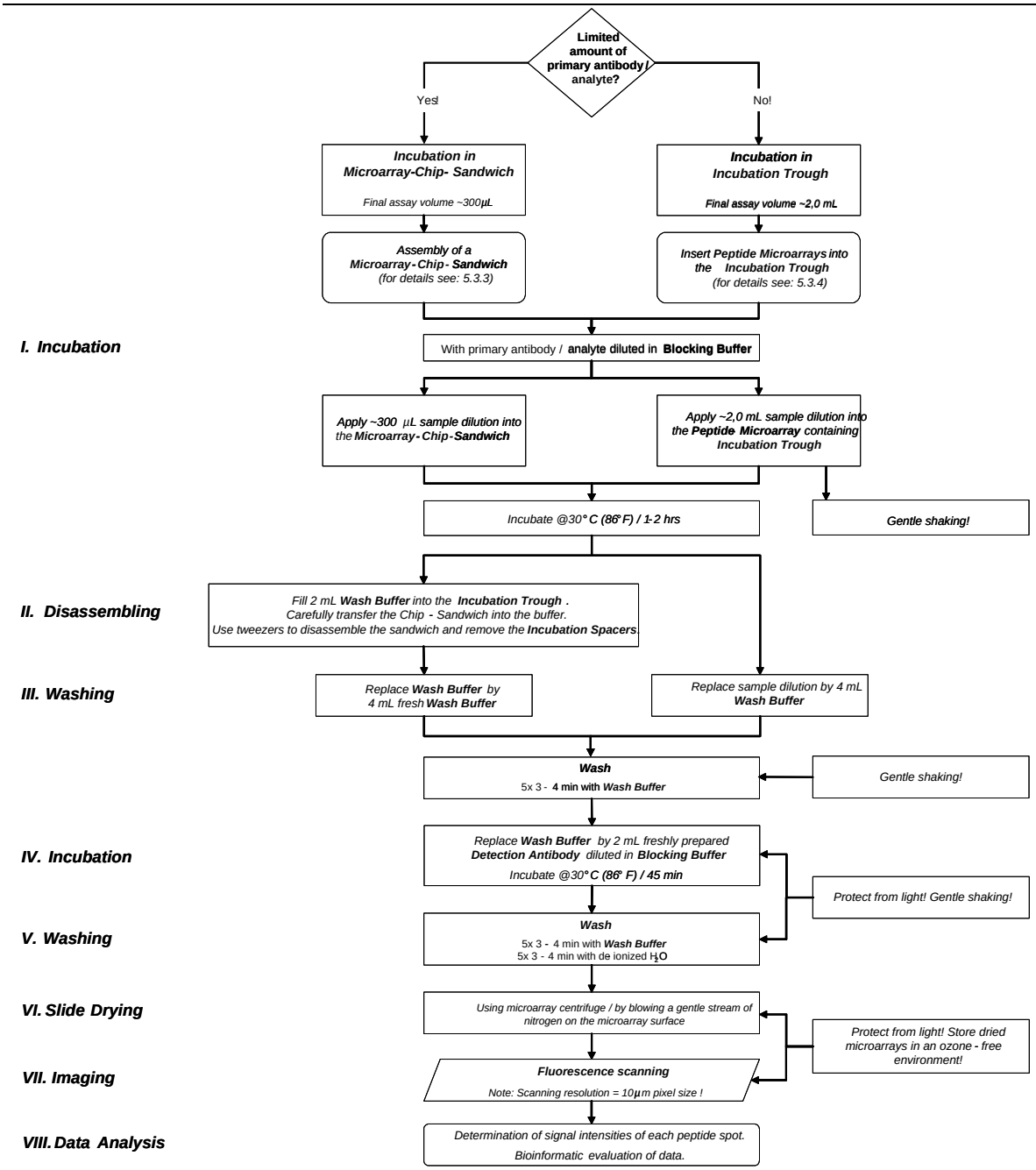


Figure 6: Exemplary method for incubation of JPT's RepliTope™ microarrays in Tecan HS4X00 processing machines:

Ch.: 1 and 2: TBS buffer, 0.1% Tween20
Ch.: 5: 0.1x SSC buffer

Step 1: Pre-wash and filling of incubation chambers
Step 2-4: Blocking procedure
Step 5-7: Incubation with primary antibody/sera
Step 8-10: Incubation with secondary antibody
Step 11: Final washing steps
Step 12: Slide drying procedure

5.3.2 Workflow for Manual Incubation of RepliTope™ Peptide Microarrays*



* For the design of a RepliTope experiment, the attached RepliTope™ Microarray Incubation Protocol (see point 6) can be used.

5.3.3 Sample incubation using microarray-chip-sandwich

To create a simple incubation chamber, two slides, one displaying the peptides and another slide (blank slide) without any peptides, have to be assembled according to Figure 7 in a sandwich-like format. If two peptide microarrays are to be screened in parallel, the top slide would be another peptide-displaying chip. Please make sure that in such a case the two peptide-displaying sides are facing each other. The two slides are separated by two spacers.

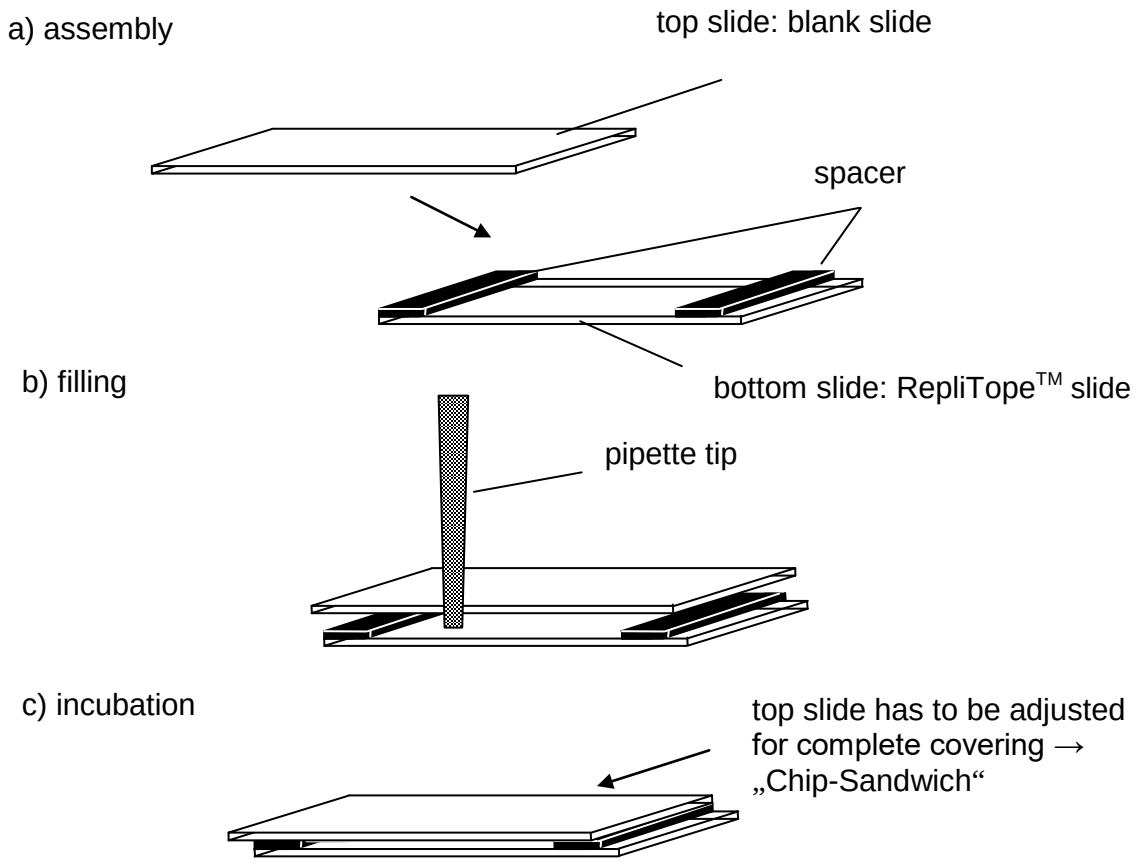
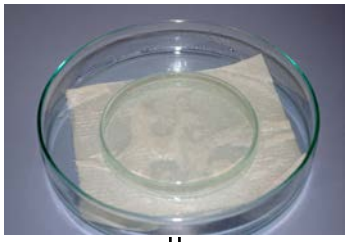


Figure 7: Assembly of a chip sandwich.
a) Two plastic spacers are placed between the peptide displaying microarray (bottom slide) and the blank slide or second peptide displaying microarray (top slide) resulting in a defined reaction chamber.
b) Assay solution is applied via pipette tip into the reaction chamber formed by the two slides. Capillary forces will soak in the solution without formation of bubbles.
c) Top microarray is shifted resulting in overlapping ends of the glass slides. This arrangement enables convenient disassembly after the incubation step.

5.3.3.1 Preparation of the Slide Environment for Easy Handling

1. **WET-CHAMBER ASSEMBLY**



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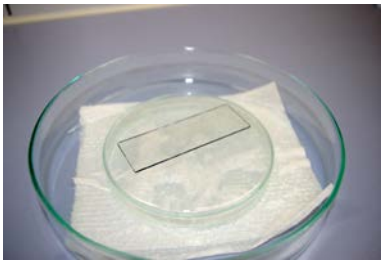
2. **PLACEMENT OF THE PEPTIDE MICROARRAY SLIDE SUPPORT PLATE FACING UPWARD**



Engraved label has to be readable from top.

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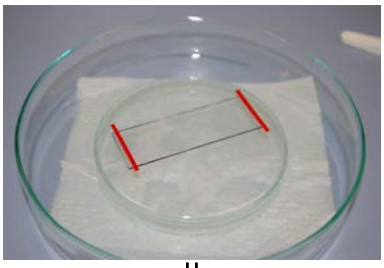
3. **PLACEMENT OF ENCLOSED SPACERS ON BOTH ENDS OF THE MICROARRAY**



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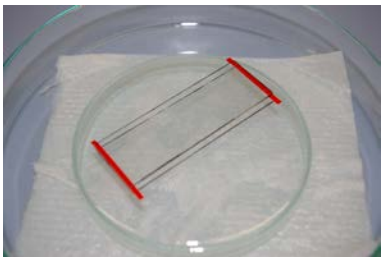
4. **ASSEMBLING OF THE MICROARRAY SANDWICH**



See also 5.3.3. If two peptide microarrays are used make sure that peptide displaying sides are facing each other.

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5. **PREPARATION OF FINAL ASSAY SOLUTION CONTAINING TARGET ANTIBODY/ANALYTE**

Approx. 300 µL if enclosed spacers are used.

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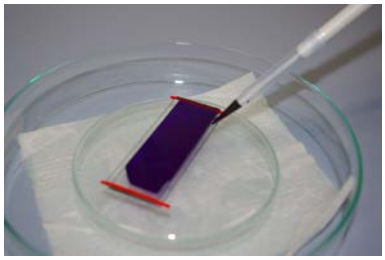
6. **PIPETTING OF THE COMPLETE VOLUME INTO MICROARRAY CHIP-SANDWICH**



Capillary forces will suck the solution in between the two slides. Avoid air bubbles within the sandwich.

⚠	Make sure not to touch the microarray with the pipette tip. Scratches and marks on the surface may destroy the deposited microarray and will cause artifacts!

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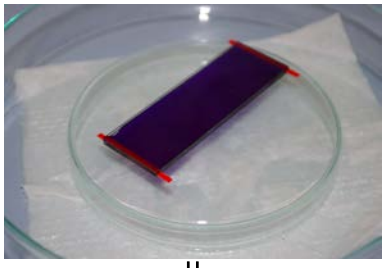
7. **ADJUSTMENT OF THE PEPTIDE MICROARRAY SANDWICH**



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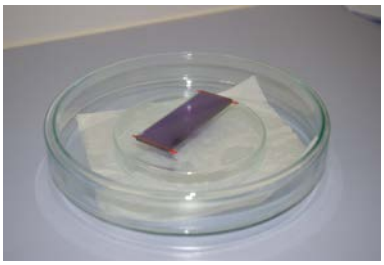
8. **CLOSING OF THE PETRI-DISH WITH A MATCHING COVER TO CREATE AN INCUBATION CHAMBER.**



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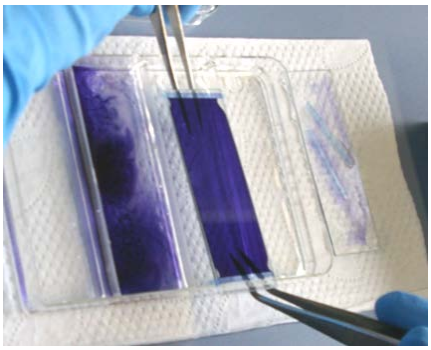
⚠	JPT does not recommend use of fluorescently labeled primary or secondary antibodies in microarray sandwich-like incubations.

9. **TRANSFER THE MICROARRAY-CHIP-SANDWICH INTO THE WASH BUFFER CONTAINING INCUBATION TROUGH AFTER SAMPLE INCUBATION IS FINISHED**



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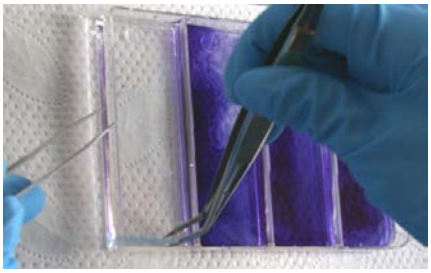
10. **REMOVING OF THE DUMMY/BLANK-SLIDE AND INCUBATION SPACERS**



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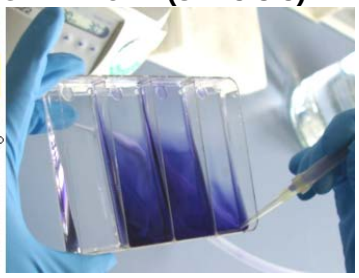
11. **THE FOLLOWING WASH AND INCUBATION STEPS ARE PERFORMED IN THE INCUBATION TROUGH UNDER GENTLE SHAKING ON A ROCKING PLATFORM (SEE 5.3.5)**

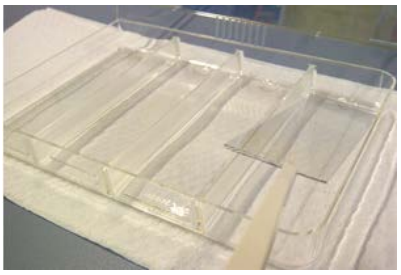


Remove sample dilutions and wash solutions by aspiration. Protect all incubation steps containing fluorescently labeled samples or antibodies from light!

Ensure that microarrays are properly washed with enough liquid rinsing over the slide. Do not allow the microarray slides to dry until the last washing step of the incubation procedure!

5.3.4 Sample Incubation using Incubation trough

1. **PLACEMENT OF THE PEPTIDE MICROARRAY SLIDES IN THE INCUBATION TROUGH**



Engraved label has to be readable from top!

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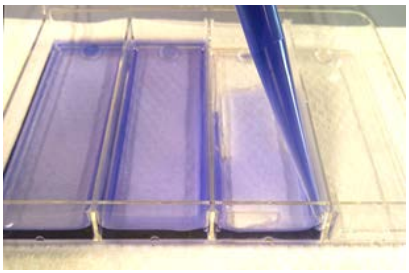
2. **ADDITION OF THE SAMPLE DILUTION**



Not directly on the microarray slide surface, but in one edge of the incubation trough!

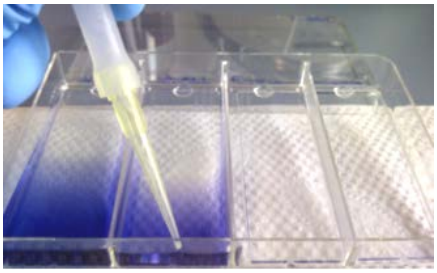
⇓

3. **CLOSE THE LID AND INCUBATE MICROARRAY SLIDES FOR THE APPROPRIATE TIME AT THE DESIRED TEMPERATURE**

Gentle shaking!

⇓

4. **REMOVE SAMPLE DILUTIONS AND WASH BUFFERS BY ASPIRATION**



Ensure that microarrays are properly washed with enough liquid rinsing over the slide. Do not allow the microarray slides to dry until the last washing step of the incubation procedure!

5.3.5 Detection Antibody Incubation and Post Processing

Independent of the method used for the sample incubation, JPT recommends to incubate the microarray slides with the fluorescently labeled detection antibody by using a incubation trough!

1. **AFTER SAMPLE INCUBATION, WASHING AND WASHBUFFER REMOVING, ADD FLUORESCENTLY LABELED ANTIBODY**

Not directly on the microarray slide surface, but in one edge of the incubation trough!

⇓

2. **CLOSE THE LID AND INCUBATE THE MICROARRAY SLIDES FOR THE APPROPRIATE TIME AT THE DESIRED TEMPERATURE**

Gentle shaking! Protect all incubation steps containing fluorescently labeled samples or antibodies from light!

⇓

3. **REMOVE LABELED ANTIBODY AND WASH BUFFERS BY ASPIRATION**

⇓

4. **PERFORM A FINAL WASH STEP WITH DEIONIZED WATER TO REMOVE ALL SALT RESIDUES**

⇓

5. **DRYING THE MICROARRAY SLIDES USING A MICROARRAY CENTRIFUGE OR BY BLOWING A GENTLE STREAM OF A NITROGEN ON THE MICROARRAY SURFACE**

⇓

6. **PERFORM FLUORESCENCE SCANS OF MICROARRAY SLIDES ACCORDING TO SCANNER TYPE AND LASER SETTINGS CORRESPONDING TO THE FLUORESCENCE LABEL OF THE DETECTION ANTIBODY**

Since fluorescence dyes are affected by direct light, ozone and other environmental conditions, please make sure to scan the slides immediately after incubation. If longer storage of incubated slides is required, please seal the slides using inert gas in a dark and dry microarray box.

5.3.6 Data Analysis

For details about application and modification of .gal files, refer to the protocol: "reading a _gal-file" enclosed to the Galviewer software.

1. Generation of a list containing signal intensities of each peptide spot by means of microarray evaluation software.
2. Calculation of the mean value for the signal intensities of spots with identical peptides (three identical spots per subarray).
3. The highest values indicate the spots displaying peptides recognized most effectively by your antibody.
4. Create heatmap or bar-plot diagram for visualization and identification of major binding sites (examples see Figure 6).

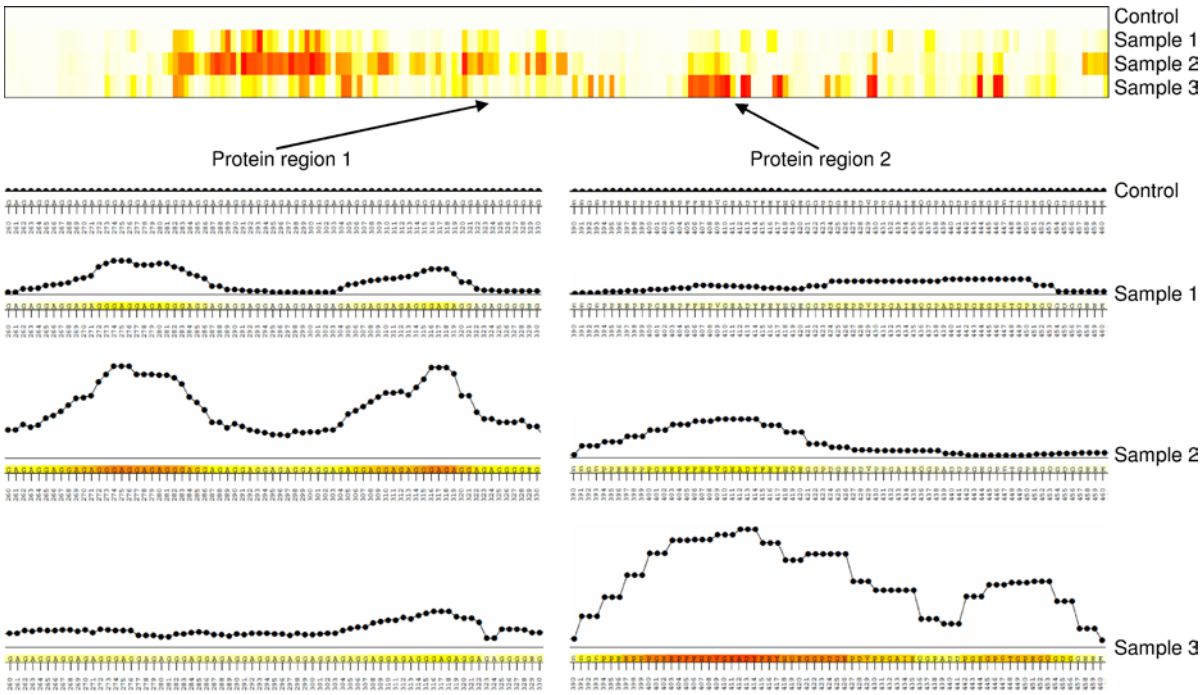


Figure 8: Microarray incubation using microarray-chip-sandwich.

The numerical data were processed using JPT's proprietary evaluation and visualization bioinformatics tools. Upper panel: visualization of results by heatmap diagram. The peptides are sorted on the x-axis according to their position in the scanned protein. Lower panel: for two regions of protein, the contribution of each individual residue to the epitope recognized was calculated using information from overlapping peptides.

6 RepliTope™ Microarray Incubation Protocol

General Information	**Experiment-#:**	
	Experiment Title:	
	RepliTope™ Name:	
	Date:	
	Operator:	
	Comments:	

	RepliTope Slide-#:				
I. Incubation	**1st Antibody / Analyte**				
	Stock concentration				
	Assay concentration				
	Diluent				
	Volume Primary Sample [µl]				
	Volume Diluent [µl]				
	Incubation Temperature				
	Incubation Time				

II.	**Disassembling**	

III.	**Washing**	
	TBS-T	

IV. Incubation	**2nd Antibody / Analyte**	
	Stock concentration	
	Assay concentration	
	Diluent	
	Volume Primary Sample [µl]	
	Volume Diluent [µl]	
	Incubation Temperature	
	Incubation Time	

V.	**Washing**	
	TBS-T	
	ddH2O	

VI.	**Slide Drying**	

VII.	**Scanning Parameters:**	
	Resolution:	
	Data management:	
	Comments:	

7 Notes / Troubleshooting

Problem	Cause	Solution
Artifacts	• Dust particles and resulting scratches	• Avoid dust or other particles during each step of the experiment • Use filtered buffers and solutions only
High background	• Nature of the sample	• Direct fluorescently labeled proteins tend to induce background signals via unspecific binding to the slide surface. Changing of buffer conditions in the incubation step can reduce background signals very efficiently • Additional washing steps can reduce non-specific binding • Variation of blocking buffers (initial blocking steps are not recommended by JPT)
	• Sample / 2nd antibody concentration	• Increased concentrations of sample / 2nd antibody may yield high background signals caused by unspecific binding to the slide surface
	• Insufficient washing	• Adjustment of washing conditions
	• Contaminated wash buffer	• All buffers and solutions should be prepared freshly every day
Saturated peptide spots	• Concentration of the 2nd antibody	• Higher dilution rates of the 2nd antibody
	• Scanning conditions	• Adjustment of scanning parameters
Unspecific signals	• Nature of the sample	• Variation of blocking buffers
	• Insufficient washing	• Adjustment of washing conditions

	• Specificity of the 2nd antibody	• Control incubations using labeled 2nd antibody alone should be performed in parallel to the actual experiment to ensure that found signals are not caused by non-specific binding of the 2nd antibody to the immobilized peptides
Little or no signals	• Incubation time	• Warranty of sufficient incubation time
	• Bleaching effects	• During the incubation step with fluorescently labeled 2nd antibody, protect the slides from light! • After application of secondary antibody keep slides in an ozone-free environment
	• Scanning conditions	• Adjustment of scanning parameters

8 Related Products

For further information visit our homepage (www.jpt.com) or contact our customer support team (peptide@jpt.com).

- PepStar™: customized peptide microarrays displaying individually synthesized peptides in triplicates on one microarray
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