RayBio® SARS-CoV-2 Spike Protein Overlapping Peptide Array

An array displaying 316 overlapping peptides representing the full-length SARS-CoV-2 Spike protein

User Manual
Version 1.1

(***Last Revised November 18, 2021***)

Catalog numbers:
PAH-SMHG-G1 (Human IgG detection)
PAH-SMHM-G1 (Human IgM detection)
PAH-SMHA-G1 (Human IgA detection)
PAH-SMMG-G1 (Mouse IgG detection)
PAH-SMRG-G1 (Rabbit IgG detection)

Please read manual carefully before starting experiment

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I. Kit Contents and Storage

1. ***Array Kit Components***

Each array kit contains the following components per 4 samples:

Item	Description	Cat. #	Size	One Glass Slide Kit
A	**Assembled Glass Slide**	SSEM-G1-4	4 sub-arrays/slide	1 slide
B	**1,000× Biotin-Conjugated Secondary Antibody**	Varies based on the catalog number. See page 6.	2 µL/vial	1 vial
C	**1,000× Cy3 Equivalent Dye Conjugated Streptavidin**	QA-CYSE	2 µL/vial	1 vial
D	**Blocking Buffer**	AA-BB-10	10 mL	1 bottle
E	**20× Wash Buffer I**	AA-WB1-30	30 mL	1 bottle
F	**20× Wash Buffer II**	AA-WB2-30	30 mL	1 bottle
G	**Adhesive Plastic Strips**		1 strip	1 strip
H	**30 ml-Centrifuge Tube**		1 tube	1 tube
I	**User Manual**	Download from www.RayBiotech.com		
J	**Peptide Sequence**			
K	**Array Map**			
L	**Analysis Tool**			
M	**Gal File**			

2. ***Storage***

Upon arrival, immediately store the entire kit at -20 ºC to -80 ºC. If stored in this manner, the kit will retain complete activity up to 6 months. For best results, thaw the kit immediately before use.

Once thawed, the kit must be used within 1 month. If the slide and reagents will not be used immediately after thawing, store the protein array glass slide (*Item A*) and Blocking Buffer (*Item D*) at -20 ºC and store all other components (*Items B, C, E, & F*) at 4 ºC (see table *below*).

Item	Description	Storage
A	**Assembled Glass Slide**	-20 °C
B	**1,000x Biotin-Conjugated Secondary Antibody**	4 °C
C	**1,000× Fluorophore-Conjugated Streptavidin**	4 °C
D	**Blocking Buffer**	-20 °C
E	**20x Wash Buffer I**	4 °C
F	**20x Wash Buffer II**	4 °C
G	**Adhesive Plastic Strips**	Room Temperature
H	**30 ml-Centrifuge Tube**	Room Temperature

3. ***Additional Materials Required***

- Distilled water
- Aluminum foil
- Small plastic boxes or containers
- Orbital shaker or oscillating rocker
- Pipettors, pipette tips and other common lab consumables
- Laser scanner for fluorescence detection (Cy3 equivalent dye)

II. Introduction

1. ***Assay Principle***

Overlapping peptides (15-mer in length, 4-mer offset) representing the full-length SARS-CoV-2 Spike protein are spotted in triplicate on a standard glass slide (25 mm x 75 mm x 1 mm), along with positive and negative controls to monitor each incubation step. When liquid sample types, such as serum or purified antibodies, are incubated on the array, “primary” antibodies in the sample will bind to their specific linear peptide epitopes. Specific primary antibody isotypes (IgG, IgM, or IgA) are then targeted by biotin-conjugated anti-IgG, IgM, or IgA secondary antibodies, respectively, that are specific for the host species.

A fluorophore-conjugated streptavidin molecule is then added, which binds to the biotin on the secondary antibody. The fluorophore enables the detection of the immobilized primary antibody via fluorescence using a laser scanner (Figure 1). The fluorescence signal is proportional to the amount of immobilized antibody. Since each spot represents a unique peptide that is known, the specific epitopes bound by the antibodies can be ascertained.

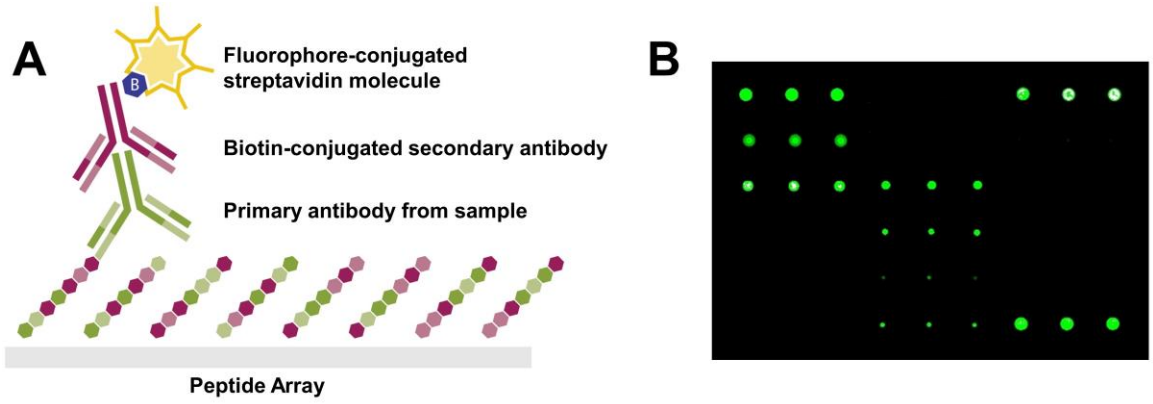


Figure 1. How peptide arrays work. A) *Schematic showing that a sample primary antibody binds to its specific epitope on the peptide array. The primary antibody is detected via a biotin-streptavidin-fluorophore complex.* ***B)*** *A representative fluorescent image of a peptide array obtained with a compatible laser scanner. Each peptide was printed in triplicate. Green spots represent primary antibody binding to the array.*

2. ***Array Overview***

Array Format	Standard glass slide (25 mm x 75 mm x 1 mm) printed with chemically synthesized peptides
Antibody Type Detected	Human IgG (Cat no. PAH-SMHG-G1) Human IgM (Cat no. PAH-SMHM-G1) Human IgA (Cat no. PAH-SMHA-G1) Mouse IgG (Cat no. PAH-SMMG-G1) Rabbit IgG (Cat no. PAH-SMRG-G1)
Array Size	4 sub-arrays per glass slide. Each sub-array can analyze 1 sample.
Detection Method	Fluorescence (Cy3 equivalent dye) with compatible laser scanner
Sample Volume	400 µL diluted sample per sub-array
Assay Duration	< 8 hours

III. General Considerations

1. ***Serum Sample Preparation***

- Negative control samples (recommended): serum samples or pooled serum pool from healthy patients to define background signals.
- If not using fresh samples, aliquot into small tubes and freeze samples at -20 °C or 80 °C.
- Avoid repeated freeze-thaw cycles.
- Avoid using hemolyzed serum or plasma as this may interfere with protein detection.
- Always centrifuge the samples (>5,000 g for 10 minutes at 4 °C) after thawing to remove any particulates that could interfere with detection. Transfer the supernatant to a new tube and keep on ice until ready to use.

2. ***Handling of Glass Arrays***

- The microarray slides are delicate. Do not touch the array surface with pipette tips, forceps, or your fingers. *Hold the slides by the edges only*. Failure to do so may negatively impact the data.
- Handle the slides with powder-free gloves and in a clean environment.
- Remove reagents/samples by gently applying suction with a pipette to corners of each chamber. Do not touch the printed area of the array. Only touch the sides of the chamber assembly (see picture on *right*).

3. ***Incubations***

- Completely cover the array area with sample or buffer during incubation steps.
- Cover the incubation chamber with adhesive strips (*Item G*) or a plastic sheet protector during incubation to avoid drying, particularly when the incubation lasts more than 2 hours or less than 70 µl of sample or reagent are used.

- During incubation and wash steps, avoid foaming and remove any bubbles from the array surface.

- Perform all incubation and wash steps under gentle rotation or rocking motion (~0.5 to 1 cycle/second).

- Avoid cross-contamination of samples to neighboring wells. To remove Wash Buffers and other reagents from chamber wells, you may invert the Glass Slide Assembly to decant into a sink or onto paper towels, and then aspirate the remaining liquid as shown in the picture on page 7.

- Several steps such as array blocking, sample incubation, biotin-conjugated antibody incubation, and fluorescence-conjugated streptavidin incubation may be done at 4 ºC overnight. Before overnight incubations, cover the incubation chamber tightly with adhesive strips (*Item G*) to prevent evaporation.

- Protect glass slides from direct light and temperatures above room temperature.

4. ***Layout of Glass Arrays***

- The RayBio® SARS-CoV-2 Spike Protein Overlapping Peptide Array is available as 4 sub-arrays per glass slide.

- The 4-subarray glass slide has no space to print a bar code. Because of this, **the lower right corner of the printed side has a tiny green mark** using a permanent marker to ensure the slide is oriented properly. However, this green mark is covered by a black frame in its assembled configuration. After removing the frame for laser scanning, the green mark can be seen on the bottom right corner if the array side is facing up. **Do not use red or black colored ink** anywhere on the slide as this may negatively affect the scanned slide image and data.

IV. Protocol

The table below describes the steps and experimental outline required to perform the array detection. The whole procedure takes ~ 8 hours.

Key Step	Action	Duration
1	Equilibrate Slide	30 min
2	Air-Dry Slide	1 hour
3	Dilute Sample	< 5 min
4	Block Array	1 hour
5	Incubate Sample on Array	1 hour
6	Wash Array	40 min
7	Incubate with Biotin-Conjugated Secondary Antibody	1 hour
8	Wash Array	40 min
9	Incubate with Fluorophore-Conjugated Streptavidin	1 hour
10	Wash Array, Dry Array, & Scan Array	1 hour

Before proceeding to the experiment, please refer to following dilution chart to prepare reagents. 1,000x Biotin-Conjugated Secondary Antibody (*Item B*) and 1,000x Fluorophore-Conjugated Streptavidin (*Item C*) should be spun down briefly (i.e., 1 – 3 seconds) to collect the contents to the bottom of the vials before dilution. Once diluted, the items should be mixed thoroughly by inverting the capped vials 6 – 10 times or vortexing the capped vials for 3 seconds.

Item	Description	Dilution Fold	Diluent	Temporary Storage	Shelf Life
B	**1,000x Biotin-Conjugated Secondary Antibody**	1,000	Blocking Buffer (Item D)	Fresh ice	Use immediately once diluted
C	**1,000× Fluorophore-Conjugated Streptavidin**	1,000	Blocking Buffer (Item D)	Fresh ice. Protect from light.	Use immediately once diluted
E	**20x Wash Buffer I**	20	Distilled water	Room temperature	1 week
F	**20x Wash Buffer II**	20	Distilled water	Room temperature	1 week

***Note**: If crystals have formed in the 20× Wash Buffer concentrates, warm the bottles to room temperature and mix gently until the crystals have completely dissolved.*

1. ***Blocking and Sample Incubation***

1.1 **Equilibrate Slide**: Take the kit package containing the Assembled Glass Slide (*Item A*) from the freezer. Place the **UNOPENED** package on the bench top for approximately 30 minutes and allow the Assembled Glass Slide to equilibrate to room temperature.

1.2 **Air-dry Slide**: Open the package carefully and take the Assembled Glass Slide (*Item A*) out of the sleeve, but *do not* disassemble the Glass Slide from the chamber assembly. Peel off the cover film and let the Assembled Glass Slide air-dry in a clean environment (e.g., fume hood) for 1 hour at room temperature.

***Note**: Protect the slide from dust and other contaminants. Incomplete drying of slides before use may cause the formation of "comet tails" of the spots.*

1.3 **Mark Slide**: If multiple slides will be tested, you will need to distinguish one slide from another. Label the front plastic frame using tiny stickers with serial numbers (*below, left*). On the back of slide, label the very top or bottom edges of glass slides using a very fine green permanent marker with the same serial numbers (*below, right*). Don't write over the printed array area, even if it is on the back unprinted side. This slide marking can also serve to orient the slide.

Note: *Permanent marker ink can significantly interfere with fluorescent signal detection. For best results during scanning, please **DO NOT***:

- Write anywhere on the front (arrayed) side of the slide
- Write on the slide while it is wet
- Write over the arrayed well areas of the slide, as this interferes with scanning
- Use red or black ink, as this interferes with scanning

1.4 **Block Array**: Add 400 µl of Blocking Buffer (*Item D*) into each well of the Assembled Glass Slide (*Item A*) and incubate at room temperature for 1 hour with gentle rocking. Ensure there are no bubbles on the array surfaces.

Note*: Be careful not to add reagents forcefully or directly to the glass slide. Always add reagents slowly along the well side.*

1.5 **Dilute Samples**: Dilute samples (for serum samples, see "Serum Sample Preparation" on page 7) in Blocking Buffer (*Item D*), and then store on ice until ready to use. *The optimal sample dilution must be determined empirically by the researcher*.

Note:

- *For human serum IgG detection, we suggest a 200-fold dilution (i.e., 2.5 µl of centrifuged serum + 497.5 µl of Blocking Buffer (Item D)).*
- *Due to pipetting error and sample loss on the tubes, it is best practice to prepare more sample (e.g., 1.2 – 1.3x) than what is calculated to add to the array surface.*
- *If bulk samples are tested, we recommend performing serial dilutions in advance.*

1.6 Decant Blocking Buffer (*Item D*) from each well completely and immediately add diluted samples.

1.7 **Incubate Samples on Array**: Load 400 µl of diluted samples into each well. Remove any bubbles from the array surfaces. Incubate arrays with gentle rocking at room temperature for 1 hour.

Note:

- *If bulk samples are tested, we recommend incubating the samples overnight at 4 ^{o}C with gentle rocking. Use the plastic adhesive strip (Item G) to seal the wells firmly.*
- *Do not let the array air dry between all steps; otherwise, it will cause high background. It is recommended to handle all slides sequentially, for example, run Steps 1.6 and 1.7 for slide A first and then repeat Steps 1.6 and 1.7 for slide B, etc.*

1.8 Decant the samples from each well

1.9 **Wash Array:** Wash the wells 5 times with 500 µl of 1× Wash Buffer I (*Item E*). Wash at room temperature with gentle shaking for 5 minutes per wash. Completely remove 1× Wash Buffer I after each wash step.

***Note**: When adding the wash buffer to the wells, avoid having the solution from one well flowing into neighboring wells.*

1.10 **Wash Array (Again):** Wash 2 times with 500 µl of 1× Wash Buffer II (*Item F*). Wash at room temperature with gentle shaking for 5 minutes per wash. Completely remove 1× Wash Buffer II after each wash step. Incomplete removal of the wash buffer may cause "dark spots" (i.e., background signal is higher than that of the spot).

2. *Biotin-Conjugated Secondary Antibody Incubation*

2.1 Briefly spin down the vial of 1,000× Biotin-Conjugated Secondary Antibody (*Item B*). Add 2 ml of Blocking Buffer (*Item D*) and mix well. Spin down.

2.2 Add 400 µl of diluted Biotin-Conjugated Secondary Antibody (*above*) into each well.

2.3 Incubate at room temperature for 1 hour with gentle shaking.

2.4 Wash with 1× Wash Buffer I as described in *Step 1.9*, then wash with 1× Wash Buffer II as described in *Step 1.10*, *above*.

3. *Fluorophore-Conjugated Streptavidin Incubation*

3.1 Add 400 µl of diluted 1x Fluorophore-Conjugated Streptavidin (diluted *Item C*) into each well.

3.2 Cover the incubation chamber with aluminum foil to avoid exposure to light or perform the incubation step in a dark room.

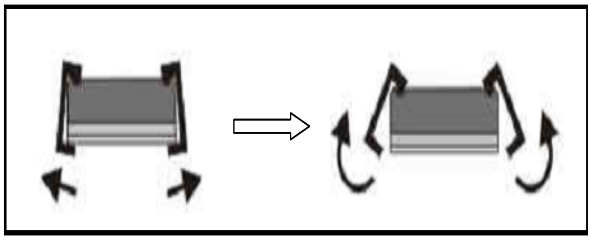
3.3 Incubate at room temperature for 1 hour with gentle rocking.

3.4 Wash with 1× Wash Buffer I as described in *Step 1.9*, then wash with 1× Wash Buffer II as described in *Step 1.10*, above.

3.5 Decant excess 1× Wash Buffer II from wells.

4. ***Fluorescence Detection***

4.1 Carefully disassemble the glass slide from the incubation frame and chamber by pushing the clips outward from the sides, as shown below. Carefully remove the glass slide from the gasket. Don't touch the printed surface of the glass slide, which is on the same side as the green mark that has been added by RayBiotech, which will be on the lower right corner (printed side up) (see also "Layout of Glass Arrays" on page 8 for more information).



4.2 Place the whole slide in the 30-ml Centrifuge Tube (*Item H*) included in the kit or a glass slide holder with the lid. Cover the tube with aluminum foil to protect the slide from light. Add enough 1× Wash Buffer I (about 30 ml) to cover the whole slide and gently shake or rock at room temperature for 15 minutes. Decant 1× Wash Buffer I.

4.3 Wash with 1× Wash Buffer II (about 30 ml) with gentle shaking at room temperature for 10 minutes. Decant 1x Wash Buffer II.

4.4 Take glass slide out of the wash container, and gently apply suction with a pipette to remove any water droplets. Do not touch the printed array area, only the unprinted area. Let the slide air-dry completely for at least 20 minutes (protect from light).

***Note**: Make sure the slides are **absolutely** dry before starting the scanning procedure or storage. High background can result from incomplete drying of the slide.*

4.5 **Scan slide**: The array signals can be visualized through use of a compatible laser scanner capable of measuring signal in the green channel (i.e., equivalent to Cy3). Scan all slides at the same PMT. It is recommended that a higher PMT is used for low signal, and a low PMT for high signal.

V. Data Analysis

1. ***Data Extraction***

The captured array signal can be extracted with most microarray analysis software packages (e.g., GenePix, ScanArray Express, ArrayVision) associated with the laser scanner. Tips in data extraction:

- Ignore any comet tails.
- Define the area for signal capture for all spots, usually 100-120 micron in diameter, using the same area for every spot.
- Use median signal value, not the total or the mean.
- Use local background correction (also median value).
- Exclude obvious outlier data in calculations.

The **GAL file**, which details the peptide spot locations for microarray analysis software packages, can be downloaded from the product page at www.RayBiotech.com

2. ***Control Systems***

Positive controls and negative controls are included on the array to assist in data normalization, array orientation determination, background evaluation, etc. These internal controls help monitor the major assay steps, normalize data, and account for background noise. The following table describes the controls included on the array and their functions.

Controls	Printed Targets	Control Function
Positive Controls	**Bio-BSA:** Biotin-Conjugated Bovine Serum Albumin	Array orientation
		Data normalization
		Evaluate the activity of the Fluorophore-Conjugated Streptavidin (Item C)
	Human IgG, IgM, and IgA; Mouse IgG; and rabbit IgG	Evaluate the activity of Biotin-Conjugated Secondary Antibodies (Item B)
	Recombinant SARS-CoV-2 Spike Subunit 1, Subunit 2, and Receptor Binding Domain	Evaluate the binding of primary antibodies to specific regions or domains of the Spike protein
Negative Controls	1× phosphate buffered saline (PBS)	Evaluate the background noise

3. ***Data Normalization***

Raw data normalization is used to compare data between sub-arrays (i.e., different samples) by accounting for the differences in signal intensities of **the positive control spots** on those arrays. The positive control (PC) is a controlled amount of biotinylated protein (Bio-BSA) that is printed on the arrays in triplicate spots. The amount of signal from each PC spot is dependent on the amount of the reporter (i.e., Fluorophore-Conjugated Streptavidin) bound to the biotinylated protein.

As such, any differences in the average signal of a set of PC spots from one sub-array to another sub-array will accurately reflect the signal differences between the sub-arrays.

To normalize the data, one array must be defined as the "**Reference Array** (*r*)" to which the signals of other "**Sample Arrays** (*s*)" are normalized. It is up to the customer to define which array should be the reference. The normalized values are calculated as follows:

$$\mathrm{n}\mathbf{X}s = \mathbf{X}s \times \frac{\mathbf{P}r}{\mathbf{P}s}$$

- **P***r*: the average signal value of all Bio-BSA spots on the reference array (*r*)
- **P*s***: the average signal value of all Bio-BSA spots on the sample array (*s*)
- **X*s***: the signal value for a particular spot (*X*) on sample array (*s*)
- **nX*s***: the normalized X*s* value. *All signals from the Sample Array (s) should be multiplied by **nXs**; the newly calculated signals are the normalized signals.*

For example, if one sub-array that was defined as the Reference Array (*r*) had a **P***r* of 40,000 and another sub-array defined as the Sample Array (*s*) had a **P***s* of 20,000, then the overall signal of the Sample Array (*s*) is half as high as the Reference Array (*r*). The equation above accounts for this discrepancy by multiplying the spot signals in the Sample Array (*s*) by 2.

4. Threshold of significant difference in expression

The background signals should be subtracted from all spots, including the negative control sample's spots. The sample spot intensities across arrays should also be normalized using the positive controls as described in "Data Normalization" above. By

comparing the signal intensities of each target between and among array images, the relative differences in expression levels of each analyte between samples or groups can be determined.

Fold differences in single analyte signals across samples that are ≥1.5 may be considered a measurable and significant difference in expression, provided that both sets of signals are well above background.

5. Analysis Tool

The extracted signal intensities from the microarray analysis software can be imported into our Excel-based SARS-CoV-2 Spike Protein Overlapping Peptide Array Analysis Tool (Cat no. PAH-SSEM-G1-SW). This analysis tool is simple and free to use; it can be downloaded from the product page. The RayBio® Analysis Tool software will not only assist in compiling and organizing your data, but it will also reduce your calculations to a simple “copy and paste” step. The Analysis Tool will help you:

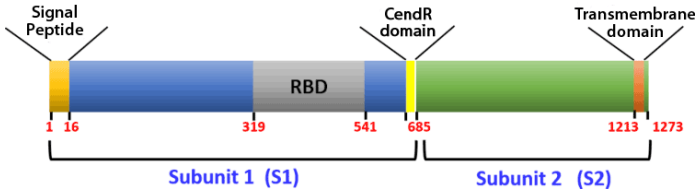
- Assign your signal intensities to the array map
- Sort the target list
- Average signal intensities
- Subtract background
- Normalize the data from different samples

VI. Appendix

1. Array Target List

A total of 316 peptides representing the full-length SARS-CoV-2 Spike protein (UniProt Accession Number: P0DTC2) (aa 1-1,273, *below*) were chemically synthesized. They were then purified by HPLC with >95% purity and confirmed with mass spectrometry. The peptides are 15 amino acids in length (15-mer), with peptides representing adjacent regions on the spike protein overlapping each other by 11 amino acids (4-mer offset).

The peptide sequences can be downloaded from the product page.



2. Array Map

Each sub-array is printed in a 33 column x 35 row format. All peptides were printed in triplicate. Positive control spots include biotinylated bovine serum albumin (Bio-BSA) and purified and glycosylated recombinant SARS-CoV-2 Spike proteins [Spike receptor binding domain (RBD) (Cat no. 230-30162), full-length Spike S1 subunit (Cat no. 230-30161), full-length Spike S2 subunit (Cat no. 230-30163)]. The negative control spots is 1x phosphate buffered saline (PBS). For more information on the control spots, see “Control Systems” on page 14.

The array map can be downloaded from the product page.

3. *Reference List*

- F Wu, et al. A new coronavirus associated with human respiratory disease in China. Nature. 579, 265–269 (2020).
- N Dong, et al. Genomic and protein structure modelling analysis depicts the origin and infectivity of 2019-nCoV, a new coronavirus which caused a pneumonia outbreak in Wuhan, China. bioRxiv (2020).
- M Hoffmann, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell. 181, 1–10 (2020).
- W Li et al. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature. 426, 450–454 (2003).

4. ***Troubleshooting Guide***

Problem	Potential Causes	Recommendation
Weak Signal	Inadequate detection	Increase laser power and/or PMT parameters
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Short incubation times	Ensure sufficient incubation time or change sample incubation to an overnight step at 4 ^{o}C
	Protein or antibody concentrations in sample are too low	Dilute sample less, concentrate sample, or add more sample volume to well. If adding more sample to well, ensure that the samples does not cross-contaminate neighboring wells.
	Improper storage of kit	Store kit at suggested temperature; Don't freeze/thaw the slide
High Background	Excess protein or antibody	Dilute protein or antibody further
	Excess streptavidin	Dilute streptavidin further
	Overexposure	Decrease the laser power
	Dust	Minimize dust in work environment before starting experiment
	Slide dried out between steps	Take additional precautions to prevent slides from drying out during experiment
	Dark spots	Completely remove wash buffer after each wash step
	Sample dilution too low	Dilute sample further
	Insufficient washing	Increase wash time and use more wash buffer. Wash slide in 1x Wash Buffer I (diluted *Item E*) overnight at 4 ^{o}C.
Uneven Signal	Bubbles formed during incubation	Handle and pipette solutions more gently; De-gas solutions prior to use
	Reagent evaporation	Cover the incubation chamber with adhesive film during incubation
	Arrays were not completely covered by reagent	Prepare more reagent and completely cover arrays with solution

Note:

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