RayBio® Human Immune Checkpoint Receptor Protein Array

An array featuring 37 essential human immune checkpoint receptor proteins, all immobilized on a single glass slide.

User Manual
Version 1.0

(***Last Revised on Dec 19, 2024***)

Catalog numbers:

PAH-ICPH-G1 (Human IgG detection)
PAH-ICPM-G1 (Mouse IgG detection)
PAH-ICPR-G1 (Rabbit IgG detection)

Please read the manual carefully before starting the experiment

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I. Kit Contents and Storage

1. *Array Kit Components*

Each array kit contains the following components per 16 samples:

Item	Description	Cat. #	Size	One Glass Slide Kit (Qty.)
A	**RayBio® Immune Checkpoint Receptor Protein Glass Array**	ICP-G1-16	16 sub-arrays/slide	1 slide
B	**1,000× Biotin-Conjugated Secondary Antibody**	Vary on kit catalog numbers. **See Page 10.**	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	**Analysis Tool**			
K	**Gal File**			

2. *Storage*

Upon arrival, the entire kit must be stored immediately at -20 ºC to -80 ºC until just before the experiment. When stored under these conditions, the kit maintains full activity for up to 6 months.

Once thawed, the kit must be used within 1 month. If the glass slide and reagents are not 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	**RayBio® Immune Checkpoint Receptor Protein Glass Array**	-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

Immune checkpoint proteins are a type of membrane protein found on some immune cell surface, such as T cells and antigen-presenting cells, and some cancer cells. These proteins play a vital role in maintaining immune homeostasis and preventing autoimmunity but are also implicated in immune evasion in cancer and other diseases.

RayBio® Human Immune Checkpoint Receptor Protein Array is a laboratory tool that allows researchers to simultaneously detect and screen the binding affinity of the antibodies, antibody drug, ligands, other binders, to the critical immune checkpoint receptor proteins, which are affinity purified and immobilized on a single treated glass slide surface, providing a snapshot of the immune response by analyzing the key regulatory proteins like PD-1, CTLA-4, TIGIT, LAG-3, and others on immune cells, often used in cancer research to study tumor microenvironment and potential targets for immunotherapy.

1. ***Assay Principle***

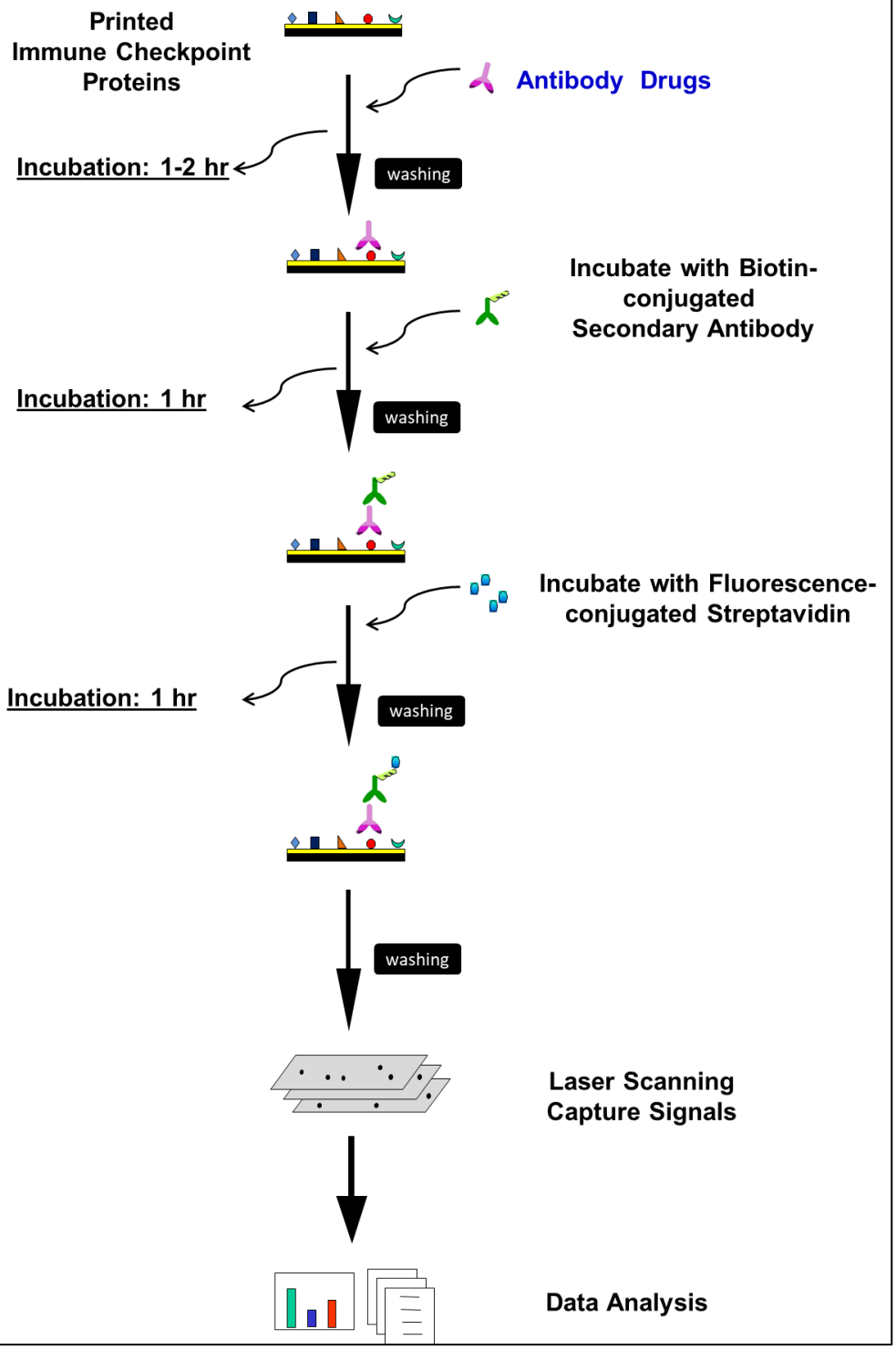
The purified receptor proteins are arrayed in triplicate on a standard glass slide (25 mm x 75 mm x 1 mm), along with positive and negative controls to monitor each incubation step. The array testing method varies depending on the type of sample applied to the array. For example, when liquid sample types, such as serum or purified antibodies, are incubated on the array, “primary” antibodies (for example, antibody drug) in the sample will bind to their specific target receptor proteins. Specific primary antibody isotypes (human, mouse, or rabbit IgG) are targeted using biotin-conjugated secondary antibodies designed for the corresponding host species (see the flow chart on the next page).

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

Depending on the types of biological samples, three typical assays (A, B, and C) are listed below.

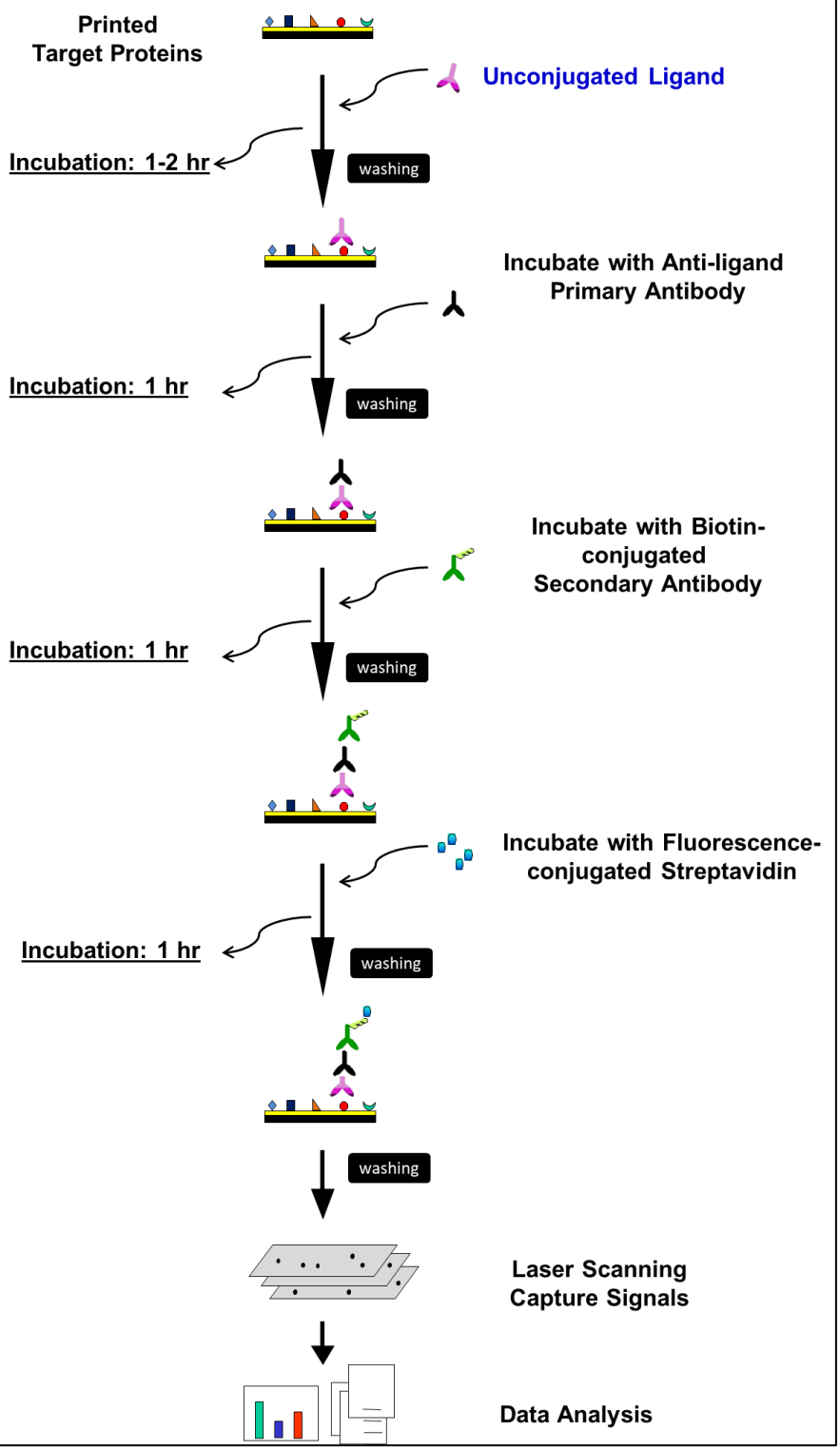
- **<u>Assay A</u>: Test the antibodies with Fc tag.** Directly incubate the array with samples. If the sample antibodies have His, Flag, or myc tags, instead of Fc-tag, an extra step of incubation with the anti-tag primary antibody is needed before the secondary antibody incubation (Figure 1).

Figure 1



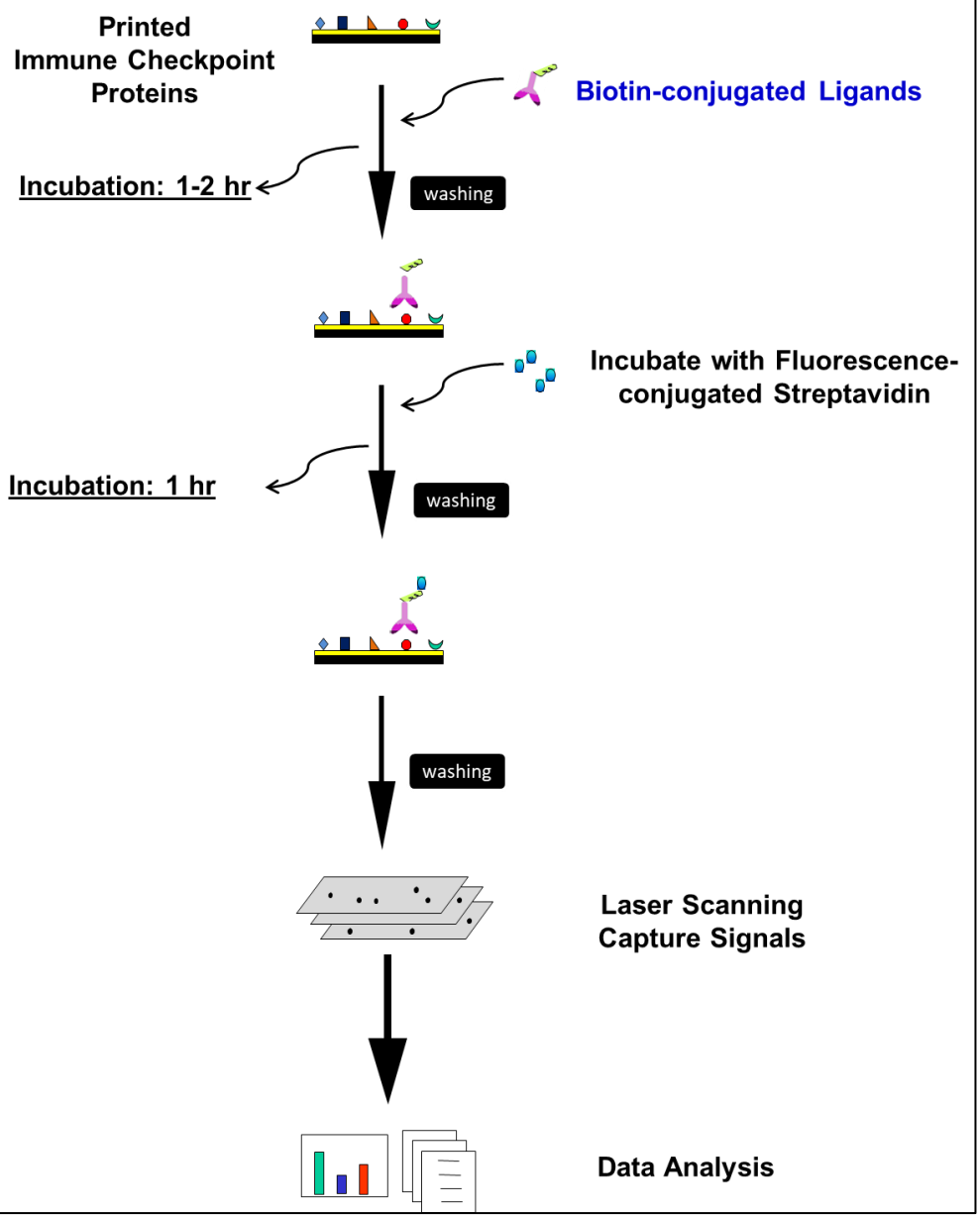
- **<u>Assay B</u>: Test the unconjugated ligands**. If the ligands are drugs, not an antibody, they have no tags, but the anti-ligand primary antibody is available by customers. After array incubation with ligands, one additional step of incubation with anti-ligand primary antibody is needed before the secondary antibody incubation (Figure 2).

Figure 2



- **Assay C: Test the biotin-conjugated ligands**. If the ligands are not an antibody, and have neither tags nor primary antibody, the easy and fast way is to conjugate the ligands with small molecule biotin (Figure 3). This method omits all antibody incubation steps. Raybio provides target biotin-conjugation service for your convenience, please contact us at tech@raybiotech.com.

Figure 3



Array Function

RayBio® Immune Checkpoint Receptor Protein Arrays are printed with 37 important immune checkpoint receptor proteins, which bind to their corresponding ligand proteins, antibodies, and other binders, allowing for simultaneous detection of multiple checkpoint receptors on a single platform.

Applications

- Detection of antibody binding to different immune checkpoint proteins from serum or plasma samples
- Therapeutic drug discovery and development: Screening and characterizing small-molecule binders, various antibody types (polyclonal, monoclonal, scFv, VHH, etc.), and biologics targeting immune checkpoints.
- Biomarker discovery: Identifying new biomarkers for immune checkpoint activity or dysfunction.
- Mechanistic studies: Understanding signaling pathways and receptor interactions in the immune system.
- Clinical research: Evaluating patient-specific responses to immune checkpoint inhibitors in cancer therapy.

Features

- Simultaneous profiling of multiple immune checkpoint proteins
- High throughput analysis
- Semi-qualitative data acquisition
- All recombinant receptor proteins were expressed in mammalian cells with abundant post-translation modifications (PTMs)
- Expressed regions are either the full-length proteins or the extracellular cellular membrane domains (ECD)
- All receptor membrane proteins are affinity-purified with high purity

2. ***Array Overview***

Array Format	Standard glass slide (25 mm x 75 mm x 1 mm) printed with 37 affinity-purified human receptor proteins
Antibody Type Detected	**PAH-ICPH-G1-16** (Human IgG detection) **PAH-ICPM-G1-16** (Mouse IgG detection) **PAH-ICPR-G1-16** (Rabbit IgG detection)
Array Size	16 sub-arrays per glass slide. Each sub-array can analyze one sample.
Detection Method	Fluorescence (Cy3 equivalent dye) with laser scanner
Sample Volume	100 µL diluted sample per sub-array
Data Type	Semi-quantitative
Assay Duration	< 8 hours

III. General Considerations

1. ***Serum Sample Preparation (if test sample is serum)***

- 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 ^{0}C) after thawing to remove any particulates that could interfere with detection. Transfer the supernatant to 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 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 is used.

- During incubation and wash steps, avoid foaming and remove any bubbles from the sub-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 and aspirate the remaining liquid as shown in picture, 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, strong light, and above room temperature.

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

- RayBio® Protein Array has 16 sub-arrays per glass slide (below). It is better to match the subarray ID numbers to your sample ID numbers. The analysis tool uses the same sample loading order (left to right, top to bottom).

1	2
3	4
5	6
7	8
9	10
11	12
13	14
15	16

- The 16-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 to you. **Do not use red or black colored ink** anywhere on the slide as this may negatively affect the scanned slide image and data.

- **Do not let array air dry between all steps;** otherwise, it will cause high background.

IV. Protocol

The table below describes the steps and experimental outline required to perform the typical array detection in Assay A (page 5). The whole procedure takes ~8 hours.

Assay B has one more step incubation than Assay A. Assay C is short and omits all antibody incubations.

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. The vials containing 1,000x Biotin-conjugated Secondary Antibody (*Item B*) and 1,000x Fluorophore-conjugated Streptavidin (*Item C*) should be spun down briefly to collect the contents to the bottom of the vial before dilution.

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

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 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 clean environment 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".*

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. See an example of 4 sub-array slide chamber 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.

Front Side Back Side

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

1.4 **Block Array**: Add 100 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 side of well.*

1.5 Dilute Samples: it depends on what types of samples are used in the methods (Figures 1-3). All samples and antibodies below are diluted with Blocking Buffer (*Item D*).

Assay A: Test the antibodies with Fc tag

1.6 Incubate Samples on Array: Decant Blocking Buffer from each well completely and immediately add 100 µl samples (for example, hybridoma culture, serum, purified antibody, scFv, etc.) into each well, diluted with Blocking Buffer (*Item D*). Remove any bubbles from the array surfaces. Incubate arrays with gentle rocking at room temperature for 1 hour. **Go to Step 1.7, page 17.**

Note:

- *Pretest with serial sample dilution is recommended to optimize the sample dilution factor.*
- *It is recommended to include control samples. For example, if testing serum is from patients, including serum from healthy controls.*
- *If bulk samples are tested, we recommend incubating the samples overnight at 4 °C with gentle rocking. Use the plastic adhesive strip (Item G) to seal the wells firmly.*

Assay B: Test the unconjugated ligands

1.6.1 Incubate ligand samples on array: Load 100 µl of serial-diluted ligand samples into each well, diluted with Blocking Buffer (*Item D*). Remove any bubbles from the array surfaces. Incubate arrays with gentle rocking at room temperature for 1 hour.

Note:

- *Pretest with serial sample dilution is recommended to optimize the sample dilution factor.*
- *It is recommended to include control samples. For example, if testing serum is from patients, including serum from healthy controls.*
- *If bulk samples are tested, we recommend incubating the samples overnight at 4 °C with gentle rocking. Use the plastic adhesive strip (Item G) to seal the wells firmly.*

1.6.2 Array washings: Decant the samples from each well.

1.6.2.1 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**:* Dilute 20× Wash Buffer I (*Item E*) to 1× with distilled water. *When adding the wash buffer to the wells, avoid having the solution from one well flowing into neighboring wells. If crystals have formed in the 20× concentrate, warm the bottles to room temperature and mix gently until the crystals have completely dissolved.*

1.6.2.2 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., the background signal is higher than that of the spot).

***Note**:* Dilute 20× Wash Buffer II (*Item F*) to 1× with distilled water. *If crystals have formed in the* 20× *concentrate, warm the bottles to room temperature and mix gently until the crystals have completely dissolved.*

1.6.3 <u>Incubate array with the anti-ligand primary antibody</u>: add 100 µl anti-ligand primary antibody into each well, diluted with Blocking Buffer (*Item D*). Remove any bubbles from the array surfaces. Incubate arrays with gentle rocking at room temperature for 1 hour. **Go to Step 1.7, page 17.**

Assay C: Test the biotin-conjugated ligands

1.6.1 <u>Incubate array with biotin-conjugated ligand</u>: add 100 µl biotin-conjugated ligand provided by the customer to array wells, pre-diluted with Blocking Buffer (*Item D*). Remove any bubbles from the array surfaces. Incubate arrays with gentle rocking at room temperature for 1 hour.

- *Pretest is recommended to optimize the sample dilution factor*
- *If bulk samples are tested, we recommend incubating the samples overnight at 4 °C with gentle rocking. Use the plastic adhesive strip (Item G) to seal the wells firmly.*

1.6.2 <u>Array washings</u>:

- **Go to Step 1.7, page 17.**

- **Skip Step 2, page 17.**
- **Continue to Step 3, page 17.**

1.7 **Array Washings**: Decant the samples from each well

1.7.1 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**:* Dilute 20× Wash Buffer I (*Item E*) to 1× with distilled water. *When adding the wash buffer to the wells, avoid having the solution from one well flowing into neighboring wells. If crystals have formed in the 20× concentrate, warm the bottles to room temperature and mix gently until the crystals have completely dissolved.*

1.7.2 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., the background signal is higher than that of the spot).

***Note**:* Dilute 20× Wash Buffer II (*Item F*) to 1× with distilled water. *If crystals have formed in the* 20× *concentrate, warm the bottles to room temperature and mix gently until the crystals have completely dissolved.*

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 100 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 1.7.1*, then wash with 1× Wash Buffer II as described in *Step 1.7.2*, *above*.

3. *Fluorophore-conjugated Streptavidin Incubation*

3.1 Briefly spin the vial containing 1,000× Cy3 Equivalent Dye-Conjugated Streptavidin (*Item C*) prior to use. Add 2 ml of Blocking Buffer (*Item D*) and mix well. Spin down.

3.2 Add 100 µl of diluted streptavidin (*above*) into each well.

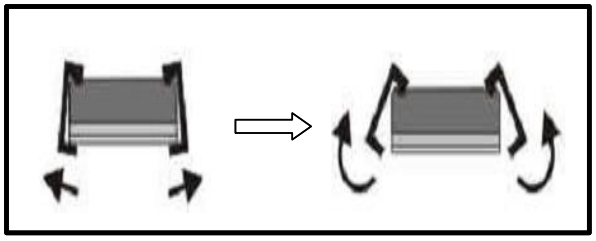
3.3 Cover the incubation chamber with aluminum foil to avoid exposure to light or perform the incubation step in a dark room.

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

3.5 Wash with 1× Wash Buffer I as described in *Step 1.7.1*. Then wash with 1× Wash Buffer II as described above in *Step 1.7.2*. 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, which will be on the lower right corner (printed side up).



4.2 Place the whole slide in 30-ml Centrifuge Tube included in the kit (*Item H*) or a glass slide holder with the lid. Cover the tube with the aluminum foil. 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 the 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 protein 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 to 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	Function
Positive Controls	**Bio-BSA:** Biotin-Conjugated Bovine Serum Albumin (BSA)	Array orientation
		Data normalization
		Evaluate the activity of the Fluorophore-Conjugated Streptavidin (*Item C*)
	Immunoglobulin (Ig): Human, mouse, rabbit IgG	Evaluate the activity of Biotin-Conjugated Secondary Antibodies (*Item B*)
Negative Controls	1× PBS	Evaluate the blank background level

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. Positive control (PC) is a controlled amount of biotinylated protein 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 biotinylated protein (Bio-BSA).

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

For example, if one sub-array that was defined as the Reference Array (*r*) had **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 for 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 Coronavirus Protein Array Analysis Tool (Cat. #. PAH-ICP-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 “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 37 mammalian cell expressed and purified human recombinant proteins were printed on glass slides.

#	Targets	CD Name
1	4-1BB, TNFRSF9	CD137
2	AIF1	
3	B7-1	CD80
4	B7-2	CD86
5	B7-H3	CD276
6	B7-H4, VTCN1	
7	B7-H5, VISTA	
8	Basigin	CD147
9	CD19	CD19
10	CD27/TNFRSF7	CD27
11	CD28	CD28
12	CD40LG, CD40L	CD154
13	CD47	CD47
14	CTLA-4	CD152
15	ICOS	CD278
16	IDO1	
17	LAG-3	CD223
18	NECTIN1	CD111
19	NECTIN2	CD112

#	Targets	CD Name
20	NECTIN3	CD113
21	OX40, TNFRSF4	CD134
22	PD-1	CD279
23	PD-L1	CD274
24	PD-L2	CD273
25	TIGIT	
26	TIM-3	CD366
27	TLR4	C284
28	TNF-alpha	
29	TNFL6	
30	TNF-R1	CD120a
31	TNF-R2	CD120b
32	TNFRSF18	CD357
33	TNFSF11, OPGL, RANKL	CD254
34	TNR11, TNFRSF11	CD265
35	TNR5, TNFRSF5	CD40
36	TNR6, TNFRSF6	CD95
37	TREM-2	

2. Array Map

Each sub-array is printed in a 12-column x 11-row format (below). All proteins were printed in triplicate. Human, mouse IgG, and rabbit IgG, biotinylated bovine serum albumin (Bio-BSA) are positive control spots whereas the 1x Phosphate Buffered Saline (PBS) are negative control (Blank) spots.

	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7	Column 8	Column 9	Column 10	Column 11	Column 12
Row 1	Bio-BSA	Bio-BSA	Bio-BSA	CD27	CD27	CD27	OX40	OX40	OX40	TNFRSF18	TNFRSF18	TNFRSF18
Row 2	Bio-BSA	Bio-BSA	Bio-BSA	CD28	CD28	CD28	PD-1	PD-1	PD-1	TNFSF11	TNFSF11	TNFSF11
Row 3	4-1BB	4-1BB	4-1BB	CD40LG	CD40LG	CD40LG	PD-L1	PD-L1	PD-L1	TNR11	TNR11	TNR11
Row 4	AIF1	AIF1	AIF1	CD47	CD47	CD47	PD-L2	PD-L2	PD-L2	TNR5	TNR5	TNR5
Row 5	B7-1	B7-1	B7-1	CTLA-4	CTLA-4	CTLA-4	TIGIT	TIGIT	TIGIT	TNR6	TNR6	TNR6
Row 6	B7-2	B7-2	B7-2	ICOS	ICOS	ICOS	TIM-3	TIM-3	TIM-3	TREM-2	TREM-2	TREM-2
Row 7	B7-H3	B7-H3	B7-H3	IDO1	IDO1	IDO1	TLR4	TLR4	TLR4	Human IgG	Human IgG	Human IgG
Row 8	B7-H4	B7-H4	B7-H4	LAG-3	LAG-3	LAG-3	TNF-alpha	TNF-alpha	TNF-alpha	Mouse IgG	Mouse IgG	Mouse IgG
Row 9	B7-H5	B7-H5	B7-H5	NECTIN1	NECTIN1	NECTIN1	TNFL6	TNFL6	TNFL6	Rabbit IgG	Rabbit IgG	Rabbit IgG
Row 10	Basigin	Basigin	Basigin	NECTIN2	NECTIN2	NECTIN2	TNF-R1	TNF-R1	TNF-R1	Blank	Blank	Blank
Row 11	CD19	CD19	CD19	NECTIN3	NECTIN3	NECTIN3	TNF-R2	TNF-R2	TNF-R2	Bio-BSA	Bio-BSA	Bio-BSA

3. ***Troubleshooting Guide***

Problem	Potential Causes	Recommendation
Weak Signal	Inadequate detection	Increase laser power and 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 °C
	Serum, protein or antibody concentrations 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 do 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	Further dilute serum, protein or antibody
	Excess streptavidin	Further dilute streptavidin
	Overexposure	Lower 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 the wash buffer after each wash step
	Insufficient washing	Increase wash time and use more wash buffer. Wash slide in Wash Buffer I (*Item E*) overnight at 4 °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 reagents and completely cover arrays with solution

4. ***Experiment Record Form***

Slide #_____ Date _____

Dilution Factor	Sample	Well No.		Sample	Dilution Factor
		1	2		
		3	4		
		5	6		
		7	8		
		9	10		
		11	12		
		13	14		
		15	16		

Note:

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