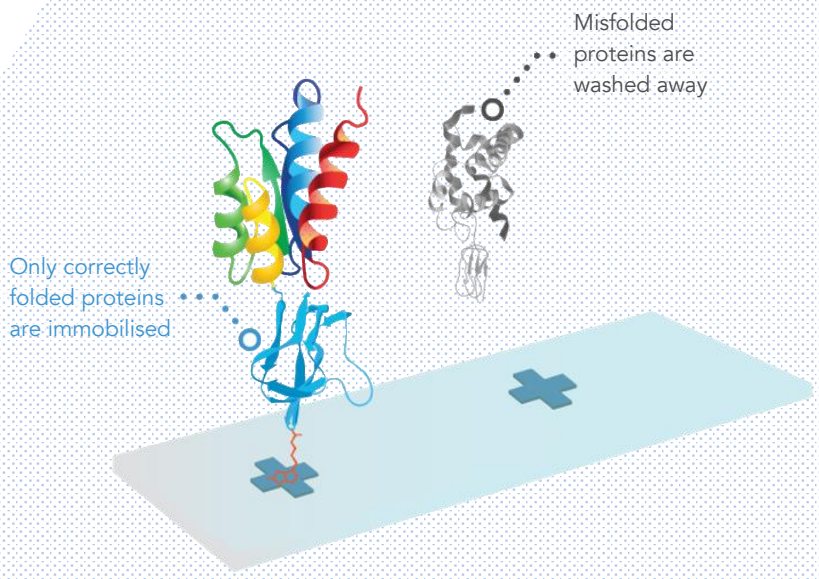
sengenics
The Functional Proteomics Company

Immunome™ Protein Array Standard Autoantibody Assay

Wet Lab Protocol

February 2021



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Contents

1.0 Purpose

This procedure defines the process to perform antibody assay on the Immunome Protein Array.

2.0 Scope

This procedure applies to all inactivated or pre-pandemic serum and plasma samples that are to be assayed on the Immunome Protein Array.

3.0 Reference Documents (Other SOPs)

3.1 Risk Assessments

1. SGN-OP-04_RA4_Autoantibody/Antibody Assay
2. SGN-OP-05_RA5_Decontamination

4.0 Relevant Personnel

Protein Microarray Lab personnel carrying out Autoantibody Assay on the Immunome Protein Array.

5.0 Definition

5.1 Inactivated samples refer to serum and plasma samples that are pre-treated with 1 % Triton X-100 for 2 hours at room temperature.

5.2 *RT*: Room temperature

6.0 Procedure

6.1 Labelling of Human IgG using PD-10 columns.

6.1.1 Required reagents, consumables and equipment

Table 1: List of reagents, consumables and equipment required for antibody labelling

REAGENTS			
Materials	**Manufacturer**	**Catalogue number**	**Storage**
Polyclonal Rabbit Anti-Human IgG	Dako	A042301	4 °C
Monoreactive Cy3-dye	GE Healthcare	GEH-PA23001	4 °C
10x Phosphate Buffer Saline (PBS)	BioSynTech	PB0344-1L	RT
Glycine	Sigma Aldrich	G8898	RT
18.2MΩcm Mili-Q Water	MiliPore	-	RT
CONSUMABLES			
PD-10 columns	GE Healthcare	17-0851-01	RT
0.5 mL non-translucent vials	Fisher Scientific	NA	RT
0.5 mL non-translucent screw cap	Fisher Scientific	NA	RT
1.5 mL microcentrifuge tube	General	NA	RT
15 mL centrifuge tubes	General	NA	RT
Gloves	General	NA	RT
10, 100 and 1000 µL tips	General	NA	RT
5 mL tips	Eppendorf	0030000978	RT
Aluminium foil	Various	NA	RT
EQUIPMENT			
NanoDrop 8000 Spectrophotometer	Thermo Scientific	ND-8000	RT
Shaker	JeioTech	SK-300	RT
5 mL pipette	Eppendorf	3120000070	RT
P10, P100 and P1000 pipette	General	NA	RT

6.1.2 PPE Required

a. Gloves
b. Lab gown
c. Mask
d. Lab shoes

6.1.3 Protocol for Labelling Human IgG using PD-10 columns

6.1.3.1 Preparation of working reagent for antibody labelling

a. Prepare 1 L of 1x PBS by adding 10 mL of 10x PBS to 900 mL of distilled water (Mili-Q water (MQH_2O)).
b. Prepare 100 mL of 1M glycine by dissolving 7.51 g of glycine powder in MQH_2O.

6.1.3.2 Determination of Anti-Human IgG concentration

a. Prepare Anti-Human IgG to determine the actual protein concentration and vortex to mix.
b. Open the spreadsheet 'TEMPLATE for Cy3-IgG concentration calculator using NanoDrop'.
c. Open the 'Protein concentration' program in the NanoDrop software. Load 1.5 µL Mili-Q water onto the pedestal to wash. Wipe it off with KimWipes.
d. 'Blank' the NanoDrop using 1x PBS. Then clean with KimWipes.
e. Load 1.5 µL of Anti-Human IgG onto pedestal A and click 'Measure'. Measure the Anti-Human IgG concentration in duplicate.
f. Save the data file in the designated file and make note of the protein concentration and absorbance at 280 nm.
g. In the excel spreadsheet 'Template for Cy3-IgG concentration calculator using Nanodrop', enter the batch numbers of Anti-Human IgG and Cy3 dye accordingly that will be used in the labelling process.
h. Enter Anti-Human IgG stock concentration given on the vial in $mg.mL^{-1}$ into the spreadsheet.
i. Enter the protein concentration and 280 nm absorbance (A280 10mm) reading into the spreadsheet to calculate the Anti-Human IgG concentration giving 1 AU.
j. Save the spreadsheet in a folder with the name of the reagent batch.
k. **Acceptance criteria**: The relative standard deviation of the determination must be <5 %.
l. Dilute down the Anti-Human IgG stock solution to 2 mg/mL by adding the required amount of 1x PBS which is given in the template.
m. Vortex thoroughly to mix after adding 1x PBS.
n. Keep the solutions on ice.

6.1.3.3 Antibody labelling

a. Open the required number of Cy3 vials and place in a tube rack.
b. Add 1 mL of the 2mg/mL Anti-Human IgG stock solution to each of the dye vials accordingly.
c. Vortex thoroughly to dissolve the dye.
d. Incubate reaction vials at room temperature for 70 minutes under aluminium foil on the orbital shaker at speed 150 rpm. Keep the reaction mixtures under foil to reduce photo bleaching.
e. Quench the labelling reactions by adding 10 µL 1M glycine to each vial. Vortex to mix and incubate on ice for 10 minutes on the shaker.

Note: PD-10 column purification can be carried out subsequently for each labelled antibody solution. Keep the remaining solutions on ice until processing.

f. Prepare five empty 1.5 mL Eppendorf tubes per PD-10 column to collect the fractions of eluate.
g. Prepare one PD-10 column by equilibrating with 25 mL 1x PBS. Drain the liquid (25 mL of 1x PBS) to the surface of the column. Caution: Do not allow column to dry out.
h. Add 1 mL of each labelling reaction to a column and allow immersing. Discard the flow through.
i. Add 1.5 mL of 1x PBS. Try to get a distinct clear band between the volumes.
j. Then further add 3.5 mL 1x PBS to flush out IgG-Cy3 from the PD-10 column. Get ready to collect the eluates in an empty 1.5 mL Eppendorf tube.
k. Start collecting the liquid as soon as it starts to become light pink in colour (Figure 1). Collect fractions of 0.5 mL volume in each 1.5 mL Eppendorf tube. Stop collecting fractions once the pink colour gets darker. Keep the fractions on ice until all the PD-10 columns have been eluted (If it involves more than one PD-10 column).

Figure 1 Distinct fraction between IgG-Cy3 and unbound Cy3

6.1.3.4 Measuring labelled protein concentration

a. Vortex the collected fractions to mix.
b. Open the 'Protein – Labelled' program in the NanoDrop software.
c. Use 1.5 µL of 1x PBS as a blank.
d. Then add 1.5 µL from tube 1 and click 'Measure'. Wipe-off sample with KimWipes and repeat this process for all the fractions that have been collected.
e. Save the data file and make note of the absorbance at 280 nm and 550 nm.
f. Open the spreadsheet 'TEMPLATE for Cy3-IgG concentration calculator using NanoDrop'.
g. Enter the protein concentration and absorbance at 280 nm and 550 nm into the spreadsheet to calculate the IgG concentration and labelling ratio.
h. If the absorbance readings are outside the range of 0.2-1.2, adjust the dilutions so that the readings fall within this range and repeat the measurements.
i. The spreadsheet calculates the concentrations and labelling ratio according to the following equations:

   i. *Antibody concentration calculation:*
   Concentration Cy3-anti-IgG (mgmL^{-1}) = (A_{280}-(0.08 x A_{552})) x Dilution x E
   Concentration giving 1 AU at 280 nm for anti-IgG = E

   ii. *Fluorophore concentration calculation:*
   *Concentration Cy3 (M)= A_{552}*Dilution/150000*
   Molar extinction coefficient at 552 nm for Cy3 dye = 150000 M^{-1}cm^{-1}.

   iii. *Recovery calculation:*
   Recovery (%) = [Volume of pooled IgG-Cy3 (µL) x average concentration (mgmL^{-1})] / [amount of antibody used in labelling reaction (µL) x unlabelled antibody concentration (mgmL^{-1})]

   iv. *Labelling ratio calculation:*
   Labelling ratio (nmole fluorophore (mg antibody)$^{-1}$) = Concentration Cy3 x 10^6 / Concentration Cy3- IgG

j. The template will help determine which of the fractions from each column should be pooled together into a 15 mL Falcon tube.

6.1.3.5 Combine fractions of Cy3-Anti Human IgG

a. Pool the desired fractions into a 15 mL Falcon tube.
b. Perform another protein concentration determination on the final pooled eluate sample and enter the readings into the template.
c. Enter the total volume of the pooled eluate sample into the template.
d. The template will determine the volume of 1x PBS to add to the pooled IgG-Cy3 to achieve the target protein concentration if needed.
e. Add the required volume of 1x PBS if necessary. Then perform a protein concentration determination on this normalized solution.
f. Enter the values for the normalized solutions into the template.

Note: The actual protein concentration should be higher than the target concentration at this point and a series of dilutions will need to be performed to achieve the target concentration.
It is better to perform these dilutions in gradual steps to avoid overshooting the target and ending up with a labelled protein at a lower target value. Approximately three dilution steps will need to be performed.

a. Enter the absorbance readings for the last dilution into the final product section of the template where the pass or fail criteria will be determined.
b. **Acceptance criteria**: The relative standard deviation of the determination must be <5 %. The recovery must be between 70 % and 125 %.
c. Aliquot 220 µL of Cy3- Anti-Human IgG using a 1000 µL pipette into 0.5 mL non-translucent vials.
d. Tap the tubes to deposit the sample at the bottom of the tube then freeze at –20 °C.

6.2 Preparation of working buffers

6.2.1 Required reagents, consumables and equipment

Table 2 List of reagents, consumables and equipment required for antibody labelling

REAGENTS			
Materials	**Manufacturer**	**Catalogue number**	**Storage**
10x Phosphate Buffer Saline (PBS)	BioSynTech	PB0344-1L	RT
Bovine Serum Albumin (BSA)	Sigma Aldrich	A3059-100G	4 °C
Triton X-100	Sigma Aldrich	T9284-100ML	RT
18.2 MΩ-cm Mili-Q Water	MiliPore	-	RT
CONSUMABLES			
Weighing boat	Various	NA	RT
50 mL tube	Various	NA	RT
5 mL tip	Eppendorf	0030000978	RT
100 µL	Various	NA	RT
EQUIPMENT			
Laboratory balancer	Mettler Toledo	JP1203C	RT
Magnetic stirrer	Heidolph	505-30000-00	RT
Magnetic stirring bar	Various	NA	RT
Spatula	Various	NA	RT
Measuring Jug, 5 L	Various	NA	RT
5 mL pipette	Eppendorf	3120000070	RT
100 µL pipette	Various	NA	RT
100 mL Beaker	Various	NA	RT

6.2.2 Preparation of working reagent

6.2.2.1 Array wash (SAB Buffer)

Table 3 Reagent composition for SAB wash buffer

Serum Albumin Buffer (SAB) – 2 Litres			
Component	% (v/v; w/v)	Volume to add (mL)	Weight of component (g)
10x Phosphate Buffer Saline (PBS)	10%	200	-
Triton X-100	0.1%	2	-
Bovine Serum Albumin (BSA)	*0.1%	-	2
Mili-Q Water (18.2MΩ)	Make up to a final volume of 2 L		

a. Collect 1.6 L of Mili-Q Water (18.2 MΩ-cm) in a clean 5 L measuring jug.
b. Add 200 mL of 10x PBS into the 5 L measuring jug.
c. Then, add 2 mL of Triton X-100.
d. Weigh out 2 g of BSA in a weighing boat using a smaller bench top balance.
e. Add the BSA to the PBS triton mixture (prepared from steps (a) to (c)).
f. Put the measuring jug on a magnetic stirrer plate and introduce a magnetic stirring bar to the jug. Begin to stir the buffer. Continue stirring until the reagents are thoroughly mixed.
g. Add Mili-Q water to make up a final volume of 2 L.
h. Store the buffer at 4 °C.

6.3 Autoantibody Assay

6.3.1 Required reagent, consumables and equipment

Table 4 List of reagents, consumables and equipment required for antibody assay

REAGENTS			
Materials	**Manufacturer**	**Catalogue number**	**Storage**
Human serum test samples	NA	NA	-20/-80 °C
Human serum control	Sigma Aldrich	H4522-20ML	-20/-80 °C
Cy3- Anti- Human IgG	In-house production	NA	-20 °C
18.2MΩcm water	NA	NA	RT
Serum assay buffer (SAB)	In-house production	NA	4 °C
CONSUMABLES			
30 mL Pap jars	Evergreen Scientific	FIS#05-557-2	RT
96 well plates (Canonical or U - bottom)	General	NA	RT
10/200/1000 µL tip	General	NA	RT
Solution Basins/Reservoir	General	NA	RT
EQUIPMENT			
Refrigerated incubator shaker	JeioTech/Medline	SI-600R	RT
Shaker	JeioTech/Medline	SK-300	RT
20 °C water bath	General	NA	RT
Vortex	General	NA	RT
Microcentrifuge 13,000 g	General	NA	RT
Microcentrifuge with MTP adapter	General	NA	RT
Multi-8 channel pipette, 200 µL	General	NA	RT
10/200/1000 µL Pipette	General	NA	RT
Pap Jar racks (24 places)	General	NA	RT
1.5 mL tube rack	General	NA	RT
Polyacetyl rack	BRAND	BR471400	RT
Polyacetyl trough	BRAND	BR471500	RT
50 mL laboratory dispenser	Various	NA	RT

Blunt forceps/spatula	General	NA	RT
Volumetric flask glass 200 mL	General	NA	RT
2 L bottle	General	NA	RT
Lab timer	General	NA	RT
MiliQ Water Purification System	General	NA	RT
Biological Safety Cabinet	General	NA	RT
Microarray scanner	Agilent Technologies	G4900DA/ G2505 C	RT
ProPlate® 2 Well Multi Array Slide System	Grace Bio-Labs	246852	RT

6.3.2 Preparation of working reagent

a. Each assay can accommodate up to 48 samples containing 47 test samples and 1 pooled normal (human serum control). One technical laboratory personnel will handle one assay at a time.
b. Pour approximately 200 mL cold Serum Albumin Buffer (SAB) into a slide trough/dish and keep at 4 °C until required.
c. Pour 2 L of SAB into a 2 L Duran bottle and equilibrate to 20 °C for 30 min in a designated circulating water bath. Fix a 50 mL laboratory dispenser to the bottle before use.
d. Pour 1 L of SAB into a 1 L Duran bottle and equilibrate to 20 °C for 30 min in a designated circulating water bath. Fix a 5 mL laboratory dispenser to the bottle before use.
e. Label the 15 mL Falcon tubes from number 1 up to 24 and place in order in a polystyrene tube rack.
f. Pipette 4.5 mL of SAB into each tube using the designated 5 mL Eppendorf laboratory dispenser.
g. Label the Quadriperm plates with consecutive numbers corresponding to the number of samples (up to 24) on the bottom of the plates.
h. Place 24 Pap jars into a suitable rack and label each tube with consecutive numbers corresponding to the number of samples (up to 24).

6.3.3 Autoantibody Assay Protocol

6.3.3.1 Sample Dilution

a. All sample dilutions must be performed in a BSCL2 cabinet.
b. Ensure all sample addition procedures are accompanied by another operator to act as a "buddy" system to ensure all samples are correctly added to the designated well as written in the sample manifest form. The second operator will observe and record all sample dilutions performed by the first operator.
c. Each slide can accommodate up to 24 samples containing 23 test samples and 1 control sample. One technical laboratory personnel can handle TWO slides at a time.
Note: You may increase the slide capacity if you feel comfortable doing so.
d. Check each sample visually to ensure that each of the tubes has sufficient serum (5.5 µL) for each assay. Place in the JeioTech shaking incubator set at 20 °C to thaw for 30 min.
e. Then, vortex mix each sample for a count of three at full speed.
f. Centrifuge for 3 minutes at 13,000 g. Disinfect the centrifuge with 70 % ethanol if spillage occurs.
g. Dilute the serum/plasma in SAB buffer to provide the assay solutions. Take the first sample, call out the sample ID on the tube, and pipette 5.5 µL of the sample into 2.2 mL of SAB Buffer in tube number 1.
h. Vortex to mix for a count of three at maximum speed. Place the vortexed tube in a different tube rack to avoid confusion with unused buffer tubes.

Note: Teammate to check that the correct samples are added to the correct tubes and mark the batch records accordingly.

6.3.3.2 Incubation with diluted samples

a. Take out the slide dish and rack containing 200 mL cold SAB.
b. According to the total slide number to be used, randomly pick Pap jars containing 2 protein array slides.
c. Take each slide in turn from their storage buffer by gripping the array between thumb and index finger at the labelled end of the slide.
d. Drain excess liquid from the slide by touching the edge of the array on the rim of the Pap jar.
e. Lift the rack from the slide dish and place the slide in slot 2 with the barcoded side facing towards slot 1. Then place the rack back in the slide dish.
f. Add each slide in turn to the rack from left to right, making sure the slides are all in the same orientation.
g. When all the slides have been added, gently shake the rack up and down five times to aid the mixing of the slide buffer interface.
h. Put the lid on the slide dish and shake on an orbital shaker at 50 rpm, for 5 minutes.

Note: Washing time that exceeds 5 minutes is not critical.

a. Place several layers of white laboratory tissue onto the bench surface and cover this with three layers of KimWipes.
b. Assemble the ProPlate® 2 Well Gasket by following the illustrated instructions in Appendix 1.

Important Note: The assembly of the ProPlate® 2 Well Gasket with a glass slide needs to be done cautiously. It needs a certain pressure to clip the gasket and the slide using the stainless-steel clip. Practice the clipping of gasket with blank glass slides first to familiarize with the pressure needed to clip the assembly and to avoid breakage of protein array slide. SAB buffer residues on glass slides will help to slide the clip smoothly.

c. Pipette 2.0 mL of each diluted sample from the 15 mL Falcon tube into their corresponding wells.
d. Place the slide immediately into an empty chamber or any container with a flat surface.
e. Set a timer to countdown for 2 hours after addition of the first array. Gently swirl the plate to cover the slide with the incubation solution.
f. After addition of all slides, scan the barcode on each slide and it will automatically log into the relevant batch record.
g. Place the cover and incubate in the shaking incubator at 50 rpm, 20 °C for 2 hours.

Note: It is advisable to assemble and process ONE slide at a time. Please ensure that sample plate and slide is in correct position.

Note: Ensure that the arrays are kept horizontal at all times to prevent slopping of solutions between wells. Handle the arrays very gently to prevent slopping or splashing of contents between wells.

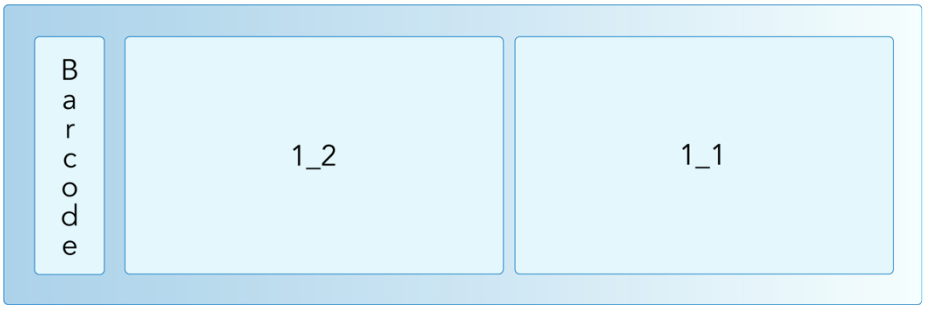


Figure 2 Sengenics Immunome Array 2-plex slide layout

6.3.3.3 Washing after Sample Incubation

a. Towards the end of the incubation period, fill Pap jars with 30 mL of SAB according to the total number of arrays.
b. **Wash 1**: When the incubation time has finished, discard the diluted samples, remove each clip from the gasket and wash each array individually in a Pap jar.
c. Cap the Pap jar and invert four times before placing in order in the Pap jar rack on the shaker and shake at 50 rpm.
d. Start a timer to countdown 20 min after addition of the first array.
e. Process the remaining slides in order and place each in the Pap jar rack on the shaker whilst shaking at 50 rpm as they are prepared.
f. **Wash 2**: After the 20 minute incubation has finished, take the first array and pour off the wash solution into an empty beaker, then dispense another 30 mL of SAB into the tube at the back of the array. Invert the Pap jar four times and place in the Pap jar rack on the shaker at 50 rpm. Start the timer to countdown 20 min.
g. **Wash 3**: When the second wash step is nearly finished, prepare a slide staining box with a rack and add 200 mL of SAB. When the second washing has finished, take the first Pap jar and pour off the buffer. Take the array between index finger and thumb and place in slot 2 of the slide rack with the barcoded side facing towards slot 1. Place the rack back in the SAB.
h. Start the time to count down 20 minutes and add the remaining arrays sequentially until all slides have been transferred. Ensure the slides are all in the same orientation and order. Replace the slide rack in buffer between the additions of each array.
i. When all the arrays have been added, gently shake the slide rack up and down five times to aid mixing. Place the lidded box on a shaker for the remainder of the incubation time at 50 rpm.

6.3.3.4 Incubation with Cy3-Anti-Human IgG

a. When the third washing step is nearly done, measure 200 mL of SAB at 20 °C into a volumetric flask, add in the Cy3-Anti-Human IgG (1:1000) solution. Mix well by repeated inversions. Pour the solution into a fresh slide staining box (without a rack) and cover until required.
b. Place a wad of KimWipes on top of the working bench. Ensure that the benches do not become contaminated with the buffer.
c. After the third wash is finished, lift the rack of arrays from the wash solution and place them on the wad of KimWipes.
d. Bang the slide rack gently on the wipes five times to remove excess wash buffer. Immediately place the arrays in the mixture of Cy3-Anti-Human IgG solution.
e. Shake the rack up and down five times to help mixing of the probing solution on the surface of the arrays. Be careful not to shake the arrays out of the racks. Set a timer to countdown for 2 hours.
f. Lid the box and shake in the shaking incubator at 50 rpm, 20 °C for the remainder of the 2 hours incubation time.

6.3.3.5 Washing after Cy3-Anti-Human IgG incubation

a. Towards the end of the incubation period, fill a slide staining box with 200 mL of fresh SAB buffer (20 °C).
b. **Wash 1**: When the incubation has finished, lift the slide rack from its incubation solution and place into the fresh SAB wash solution.
c. Shake the rack gently up and down five times. Replace the lid and shake for 5 min at 50 rpm at room temperature.
d. **Wash 2**: After wash 1 has finished, lift the slide rack out of the dish and pour off the buffer into a beaker. Pour in 200 mL of fresh SAB buffer (20 °C).
e. Shake the rack gently up and down five times. Replace the lid and shake for 5 min at 50 rpm at room temperature.
f. **Wash 3**: After wash 2 has finished, lift the slide rack out of the dish and pour off the buffer into a beaker. Pour in 200 mL of fresh SAB buffer (20 °C).
g. Shake the rack gently up and down five times then replace the lid and shake for 5 min at 50 rpm at room temperature.
h. When the third wash has finished, lift the slide rack out of the dish and pour off the SAB. Fill the box with high purity water.
i. Place the slide rack in the water and shake gently up and down five times. Then pour off the high purity water. Repeat this step three times to ensure the buffer components are washed away from the slide rack and arrays.
j. Place a wad of KimWipes on a clean bench and also in a clean and dry staining box.

k. Remove the slide rack from the dish and bang gently five times on the wad of KimWipes to remove excess water.
l. Place the slide rack in a fresh staining box on top of the KimWipes.

6.3.3.6 Drying down the slides

a. Lid the box and dry the arrays by centrifugation for 4 minutes at 400 g.

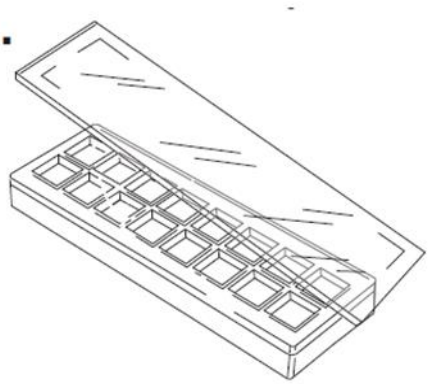
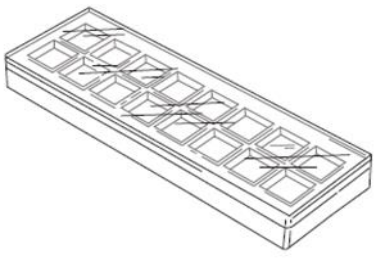
Note: Add a balancing box if necessary.

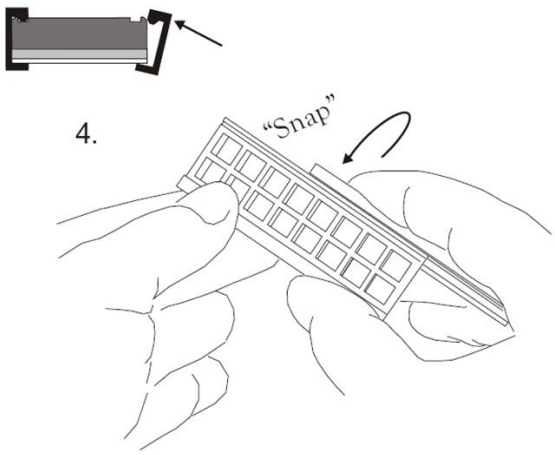
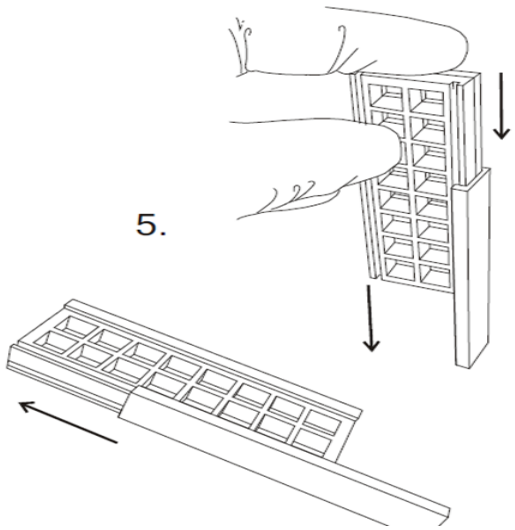
6.3.3.7 Scanning

a. Place the slides in the slide holder with the barcoded side facing upwards. Close and lock the cassette lid.
b. Place the slide holder into the Agilent slide carousel.
c. Scan the slides at 10 µm resolution, 16-bit.

Appendix 1

Illustrated instructions to assemble the ProPlate® Slide Module (e.g. showed in image below is based on a 16-well gasket. However, the method is applicable to 2-, 4-, 8- and 24-well gaskets)

1. 	Remove release liner to expose silicone gasket.
2. 	Place Sengenics ImmuSAFE COVID+ Protein Array (barcoded-side facing down) over the silicone gasket, aligning edges of the slide with the edges of the upper structure.
3. 	Press gently on the back side of the slide to adhere slide to gasket.

	Place stainless steel clip onto the slide assembly by snapping onto the long edge of the module. Grasping the assembly, place the flat inner edge of the clip over the glass slide and press the clip into the groove in the upper structure surface.
	Slide clip with the assembly until end. Alternatively, clips may be pressed against the bench top to facilitate application. Repeat assembly step for each gasket.