Immunome™ Protein Array

Autoantibody Assay

Wet Lab Protocol

April 2020

Reagent Preparation 1: Preparation of Labeled Anti-Human IgG Using PD-10 Desalting Columns

1 Chemicals

Materials	Manufacturer	Catalog number	Storage
Polyclonal Rabbit Anti-Human IgG	Dako	A042301	4°C
Monoreactive Cy3-dye	GE Healthcare	GEH-PA23001	4°C
10X Phosphate Buffer Saline	BioSynTech	PB0344-1L	Room temperature
Glycine	Sigma Aldrich	G8898	Room temperature
18.2MΩcm Mili-Q Water	MiliPore	-	Room temperature

2 Consumables

Materials	Manufacturer	Catalog number
PD-10 columns	GE Healthcare	17-0851-01
0.5 ml non-translucent vials	Fisher Scientific	NA
0.5 ml non-translucent screw cap	Fisher Scientific	NA
1.5 ml microcentrifuge tube	Various	NA
15 ml centrifuge tubes	Various	NA
Gloves	Various	NA
10, 100 and 1000 µl tips	Various	NA
Aluminium foil	Various	NA

3 Equipment

Materials	Manufacturer	Catalog number
NanoDrop 8000 Spectrophotometer	Thermo Scientific	ND-8000
Shaker	Jeiotech	SK-300

4 Pre-cautions

1. Keep Polyclonal Rabbit Anti-Human IgG on ice where possible
2. Keep the reaction mixtures (Cy3 – anti-human IgG) dark (under aluminium foil) to reduce photo bleaching.
3. Do not allow the desalting columns to dry out at the surface as this hinders flow through to column.
4. Protein solutions should only be freeze-thawed once.

5 Procedure

A. Chemical preparation before starting

Stock solution	Preparation	Storage
1X PBS	Mix 100 ml of 10X PBS and 900 ml Mili-Q water	Room temperature
1M Glycine	Dissolve 7.51 g glycine in Mili-Q water to a final volume of 100 mL	4°C. Please use within 1 month

B. Measuring anti-human IgG concentration

1. Prepare anti-human IgG to determine the actual protein concentration and vortex to mix.
2. Open the spreadsheet 'TEMPLATE for Cy3-IgG concentration calculator using NanoDrop'.
3. Open 'Protein concentration' program in NanoDrop software. Load 1.5 µl Mili-Q water on pedestal to wash. Wipe it off with KimWipes.
4. 'Blank' the NanoDrop using 1X PBS. Then clean with KimWipes.
5. Load 1.5 µl of anti-human IgG onto pedestal A and click 'Measure'. Measure the anti-human IgG concentration in duplicate.
6. Save the data file in the designated files and make a note of the protein concentration and absorbance at 280 nm.
7. In the excel spreadsheet 'Template for Cy3 IgG concentration calculator using Nanodrop', enter the batch numbers of anti-human IgG and Cy3 dye that will be used in the labelling process.
8. Enter anti-human IgG stock concentration given on the vial in $mg.ml^{-1}$ into the spreadsheet.
9. Enter the protein concentration and 280 nm absorbance (A280 10mm) reading into the spreadsheet to calculate the IgG concentration giving 1 AU.
10. Save the spreadsheet in a folder with the name of the reagent batch.
11. Acceptance criteria: The relative standard deviation of the determination must be <5%.
12. Dilute down the IgG stock solution to 2 mg/ml by adding the required amount of 1X PBS which is given in the template.
13. Vortex thoroughly to mix after adding 1X PBS.
14. Keep the solution on ice.

C. Antibody labelling

1. Open the required number of Cy3 vials and place in a tube rack.
2. Add 1 ml of the 2mg/ml IgG stock solution to each of the dye vials.
3. Vortex thoroughly to dissolve the dye.
4. Incubate reaction vials at room temperature for 70 minutes under aluminium foil on the orbital shaker at speed 150. Keep the reaction mixtures under foil to reduce photo bleaching.
5. 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.

6. Prepare five empty 1.5 ml Eppendorf tubes per PD 10 column to collect the fractions of eluate.
7. Prepare one PD-10 columns by equilibrating with 25 ml 1X PBS. Drain the liquid (25 ml of 1X PBS) to the surface of the columns.

   Precaution: Do not allow column from drying out.
8. Add 1ml of each labelling reaction to a column and allow immersing. Discard the flow through.
9. Add 1.5 ml of 1X PBS. Try to get a distinct clear band between the volumes.
10. 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 empty 1.5 ml Eppendorf tubes.
11. Start collecting the liquid as soon as it starts to become light pink in colour (Figure 1). Collect fraction of 0.5 ml volume in each of 1.5 ml Eppendorf tube. Stop collecting fractions once the pink colour gets darker.

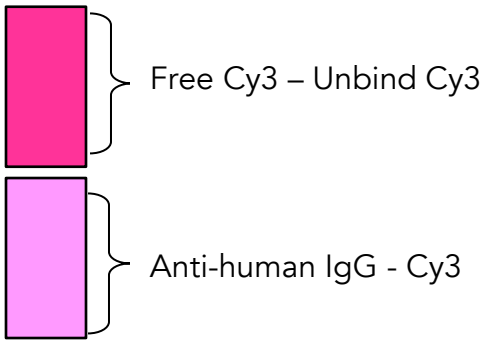

Figure 1: Distinct fraction between IgG-Cy3 and unbind Cy3.

12. Keep the fractions on ice until all the PD 10 columns have been eluted (If it is involved more than one PD 10 columns).

D. Measuring labeled protein concentration

1. Vortex the collected fractions to mix.
2. Open 'Protein -Labeled' program in NanoDrop software.
3. Use 1.5 µl of 1X PBS as a blank.
4. 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.
5. Save the data file and make a note of the absorbance at 280 nm and 550 nm.
6. Open the spreadsheet 'TEMPLATE for Cy3-IgG concentration calculator using NanoDrop'.
7. Enter the protein concentration and absorbance at the 280 nm and 550 nm into the spreadsheet to calculate the IgG concentration and labelling ratio.
8. 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.

The spreadsheet calculates the concentrations and labelling ratio according to the following equations:

a) Antibody concentration calculation:

Concentration Cy3-anti-IgG ($mgml^{-1}$)= (A_{280}-(0.08 x A_{552})) x Dilution x E

Concentration giving 1AU at 280nm for anti-IgG = E

b) Fluorophore concentration calculation:

Concentration Cy3 (M)= A_{552}*Dilution/150000

Molar extinction coefficient at 552nm for Cy3 dye = 150000 $M^{-1}cm^{-1}$.

c) Recovery calculation:

Recovery (%) = [Volume of pooled IgG-Cy3 (µl) x Average concentration ($mgml^{-1}$)] / [amount of antibody used in labeling reaction (µl) x unlabeled antibody concentration ($mgml^{-1}$)]

d) Labeling ratio calculation:

Labeling ratio (nmole fluorophore (mg antibody)$^{-1}$) = Concentration Cy3 x 10^6/ Concentration Cy3-anti-IgG

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

E. Combine fractions of Cy3 – Anti-human IgG

1. Pool the desired fractions into a 15ml Falcon Tube.
2. Perform another protein concentration determination as step 27 until 30 on the final pooled eluate sample and enter the readings into the template.
3. Enter the total volume of the pooled eluate sample into the template.
4. The template will determine the volume of 1X PBS to add to the pooled IgG-Cy3 to achieve the target protein concentration if needed.
5. Add the required volume of 1xPBS if necessary. Then perform a protein concentration determination on this normalized solution.
6. Enter the values for the normalized solution into the template.

Notes: 1. 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.

2. It is better to perform these dilutions in gradual steps to avoid overshooting the target and ending up with a labeled protein at a lower target value. Approximately three dilutions steps will need to be performed.

7. 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.
8. Acceptance criteria: The relative standard deviation of the determination must be <5%. The recovery must be between 70 % and 125 %.
9. Aliquot 220 µl of Cy3- anti IgG using a 1000 ul pipette into 0.5 ml non-translucent vials.
10. Tap the tubes to deposit the sample at the bottom of the tube then freeze at –20 °C.

Reagent Preparation 2: Preparation of Serum Albumin Buffer (SAB)

1 Chemicals

Materials	Supplier	Code	Storage
10X Phosphate Buffer Saline (PBS)	BioSynTech	PB0344-1L	Room temperature
Bovine Serum Albumin (BSA)	Sigma Aldrich	A3059-100G	4°C
Triton X100	Sigma Aldrich	T9284-100ML	Room temperature
18.2 MΩ-cm Mili-Q Water	MiliPore	-	Room temperature

2 Consumables

Materials	Manufacturer	Catalog number
Weighing boat	Various	NA
5 mL tip	Eppendorf	0030000978

3 Equipment

Materials	Manufacturer	Model
Laboratory Balancer	Mettler Toledo	JP1203C
Magnetic stirrer	Heidolph	505-30000-00
Magnetic stirring bar	Various	NA

Spatula	Various	NA
Measuring Jug, 5 L	Various	NA
5 mL pipette	Eppendorf	3120000070

4 Procedure

Serum Assay Buffer – 3 Litres			
Component	% (v/v; w/v)	Volume to add (mL)	Weight of component (g)
10X Phosphate Buffer Saline (PBS)	10%	300	-
Triton X100	0.1%	3	-
Bovine Serum Albumin (BSA)	*0.1%	-	3
Mili-Q Water (18.2MOhm)	Make up to a final volume of 3 L		

*Proportion of weight to volume (w/v)

1. Collect 2.6 L of Mili-Q Water (18.2 MΩ-cm) in a clean 5 L measuring jug.
2. Add the 300 mL of 10X PBS into the 5 L measuring jug.
3. Then, add 3 mL of Triton X100.
4. Weigh out 3 g of BSA in a weighing boat using a smaller bench top balance.
5. Add the BSA to the PBS triton mixture (prepared from steps 1 to 3).
6. Put the measuring jug on magnetic stirrer plate and introduce magnetic stirring bar to the jug. Begin to stir the buffer. Continue stirring until the reagents are thoroughly mixed.
7. Add Mili-Q water to make up to a final volume of 3 L.
8. Store the buffer at 4°C.

Autoantibody Assay Run Protocol

1 Chemicals

Materials	Manufacturer	Catalog number	Storage
Human serum test samples	NA	NA	-20/-80°C
Human serum control	Sigma Aldrich	H4522-20ML	-20/-80°C
Cy3-rabbit anti-human IgG	In-house production	NA	-20°C
18.2MΩcm water	NA	NA	Room temperature
Serum assay buffer (SAB)	In-house production	NA	4°C

2 Consumables

Materials	Manufacturer	Catalog number
Quadriperm dishes	Greiner Bio-One	96077307
30 ml Pap jars	Evergreen Scientific	FIS#05-557-2
15 ml polypropylene centrifuge tubes	General	NA
5 ml tip	Eppendorf	30000978
200/1000 ul tip	General	NA
10 ul tip	General	NA

3 Equipment

Materials	Manufacturer	Catalog number
Refrigerated incubator shaker	Jeiotech/Medline	SI-600R
Shaker	Jeiotech/Medline	SK-300
20C Waterbath	General	NA
Vortex	General	NA
Microcentrifuge 13,000 g	General	NA
Microcentrifuge with MTP adapter	General	NA
Wireless Barcode Scanner	General	NA
Pap Jar racks (24 places)	General	NA
15 ml tube rack	General	NA
Polyacetyl rack	BRAND	BR471400
Polyacetyl trough	BRAND	BR471500
10-50 ml laboratory dispenser	Various	NA
1-5 ml laboratory dispenser	Various	NA
Blunt forceps/spatula.	General	NA
Volumetric flask glass 200 ml	General	NA
5 ml Pipette	Eppendorf	3120000070
200/1000 ul Pipette	General	NA
10 ul Pipette	General	NA
Lab Timer	General	NA
MiliQ Water Purification System	General	NA
Biological Safety Cabinet	General	NA
Microarray Scanner	Agilent Technologies	G4900DA G2505 C

4 Before Started

1. Each assay can accommodate up to 24 samples containing 23 test samples and 1 pooled normal (human serum control). One technical laboratory personal will be handled one assay at a time.
2. Pour approximately 200 mL cold Serum Albumin Buffer (SAB) into a slide trough/dish and keep at 4°C until required.
3. Pour 2 litres 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.
4. Pour 1 litre 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.
5. Label the 15 mL Falcon tubes from number 1 up to 24 and place in order in a polystyrene tube rack.
6. Pipette 4.5 mL of SAB into each tube using the designated 5 mL Eppendorf laboratory dispenser.
7. Label the Quadriperm plates with consecutive numbers corresponding to the number of samples (up to 24) on the bottom of the plates.
8. Place 24 Pap jars into a suitable rack and label each tube with consecutive numbers corresponding to the number of samples (up to 24).

5 Procedure

A. Samples Dilution Preparation

1. Check each sample visually to ensure that each of the tubes has sufficient serum (11.25µL) for assay. Place in the Jeio Tech shaking incubator set at 20°C to thaw for 30 mins.
2. Then, vortex mix each sample for a count of three at full speed.
3. Centrifuge for 3 minutes at 13,000 g. Disinfect the centrifuge with 70% ethanol if spillages occur.
4. 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 11.25 µL of the sample into 4.5 mL of SAB Buffer in tube number 1.

   Note: Teammate to write down the sample ID in batch record sheets. This step must be carried out in a Class II Biological Safety Cabinet. Be careful not to disturb the undiluted serum samples during movement.
5. 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.
6. Repeat step 6 and 7 for the remaining samples.

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

B. Incubation with diluted samples

1. Take out the slide dish and rack containing 200 mL cold SAB.
2. Randomly pick 12 pap jars containing 2 protein array slides.
3. Take each slide in turn from their storage buffer by gripping the array between thumb and index finger at the labeled end of the slide.
4. Drain excess liquid from the slide by touching the edge of the array on the rim of the pap jar.
5. 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.
6. Add each slide in turn to the rack from left to right, making sure the slides are all in the same orientation.
7. When all the arrays have been added, gently shake the rack up and down five times to aid mixing at the slide buffer interface.
8. Put the lid on the slide dish and shake on an orbital shaker at 50 rpm, for 5 minutes.

   Note: Washing times exceed 5 minutes are not critical.
9. Pipette 4.0 mL of each diluted sample from the 15 mL Falcon tubes into their corresponding numbered Quadriperm chambers.
10. Place several layers of white laboratory tissue onto the bench surface and cover this with three layers of Kimwipe tissues.
11. When the slides have finished washing, remove each array in order from the rack.

    Note: Always place the rack back in the buffer between removing each array.
12. Grip the slide at the labeled end between index finger and thumb and wipe the back of the slide once on the Kimwipe tissue. Then blot the long edge of the array three times on the wad of lint free tissue paper.

13. Place the slide immediately into Quadriperm chamber containing the diluted serum sample. Ensure that the array does not rest on the lugs at the bottom of the plate.
14. Set a timer to countdown for 2 hours after addition of the first array. Gently swirl the plate to cover the slide with incubation solution.
15. After addition of all slides, scan the barcode on each slides and it will automatically log into the relevant batch record.
16. Incubate all the Quadriperm dishes in the shaking incubator at 50 rpm, 20°C for 2 hours.

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

C. Washing after Samples Incubation

5. Towards the end of the incubation period, fill 24 Pap jars with 30 mL of SAB.
6. When the incubation time has finished, remove each array from the Quadriperm dishes and wash each array individually in a Pap jar.
7. Wash 1: Use forceps or index finger to lift the array at the numbered side of the dish.

   Grip the labeled end of the slide between finger and thumb and place the first slide into its respective number of Pap jar.

   Note: Make sure that your gloves are scrupulously clean before handling arrays directly.
8. 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.
9. Start a timer to countdown 20 min after addition of the first array.

10. 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.
11. Wash 2: After the 20 minutes 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.
12. Wash 3: When the second wash step is nearly finished, prepare a slide staining box with 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 the slot 2 of the slide rack with the barcoded side facing towards slot 1. Place the rack back in the SAB.
13. Start the time to count down 20 min 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.
14. 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.

D. Incubation with Cy3-Anti-Human IgG

1. Measure 200 mL of **SAB** at 20°C into a volumetric flask and add 200 µL of Cy3-antihuman IgG. Mix well by repeated inversion. Pour the solution into a fresh slide staining box (without the rack) and cover until required.
2. Place a wad of Kimwipe tissues on top of the working bench. Ensure that the benches do not become contaminated with buffer.

3. After the third wash is finished, lift the rack of arrays from the wash solution and place on the wad of Kimwipe tissues.
4. Bang the slide rack gently on the tissues five times to remove excess wash buffer. Immediately place the arrays in the Cy3-antihuman IgG solution.
5. Shake the rack up and down five times to help mixing of the probing solution at the surface of the arrays. Be careful not to shake the arrays out of the racks. Set a timer to countdown for 2 hours
6. Lid the box and shake in the shaking incubator at 50 rpm, 20°C for the remainder of 2 hours incubation time.

E. Washing after Cy3-Anti-Human IgG incubation

1. Towards the end of the incubation period, fill a slide staining box with 200 mL of fresh SAB buffer (20°C).
2. Wash 1: When the incubation has finished, lift the slide rack from its incubation solution and place into the fresh SAB wash solution.
3. Shake the rack gently up and down five times. Replace the lid and shake for 5 min at 50 rpm at room temperature.
4. 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).
5. Shake the rack gently up and down five times. Replace the lid and shake for 5 min at 50 rpm at room temperature.
6. 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).

7. Shake the rack gently up and down five times then replace the lid and shake for 5 min at 50 rpm at room temperature.
8. 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.
9. 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.
10. Place a wad of Kimwipe tissue on the clean bench and also in the clean and dry staining box.
11. Remove the slide rack from the dish and bang gently five times on the wad of Kimwipe tissue to remove excess water.
12. Place the slide rack in the fresh staining box on top of the Kimwipe tissues.

F. Drying down the slides

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

   Note: Add a balancing box if necessary.

G. Scanning

1. Place the slide in the slide holder with the barcoded side facing upward. Close and lock the cassette lid.
2. Place the slide holder into the Agilent slide carousel.
3. Scan the slides at 10µm resolution, 16-bit.