Modified Rapid MAIPA Protocol

This method is based on the following publication;

K Campbell, K Rishi, G Howkins, D Gilby, R Mushens, C Ghevaert, P Metcalfe, WH Ouwehand, G Lucas. A modified fast MAIPA for the detection of HPA antibodies: a multi-centre evaluation of a rapid monoclonal antibody specific immobilisation of platelet antigen (MAIPA) assay. *Vox Sanguinis* 2007, **93**, 289-297.

Reagents

Coating Buffer

Na_2CO_3 1.59 g

$NaHCO_3$ 2.93 g

dH_2O to 1 litre, adjust pH to 9.6 using 0.5M HCl. Store at 4^oC.

Goat Anti-Mouse IgG

'Jackson Immunoresearch' goat anti-mouse IgG, Fcγ fragment specific (affinity purified/minimum cross reactivity) (catalogue code;115-005-164). Store at 4^oC

Dilute 1 in 500 in coating buffer. Discard any un-used diluted reagent

10X TBS Stock Buffer

Tris 12.1g

NaCl 85g

dH_2O 900mL

adjust pH to 7.4 with 0.5M HCl and make up to IL with dH_2O. Store at 4^oC

20% Bovine Serum Albumin

Bovine Serum Albumin (Sigma A-7030) 20g

Phosphate Buffered Saline 100mL. Store at 4^oC

TBS/BSA Buffer

10X TBS Stock Buffer 50mL

dH_2O 445mL

20% BSA 5mL Store at 4^oC

Solubilisation Buffer

Make 1L isotonic saline by dissolving 9.0g NaCl in 1 litre deionised water

Dissolve 1.21g Tris in 950 mL isotonic saline, pH to 7.4 using 0.5M HCl. Add 5 mL Triton X100 and mix well. Make up to 1 litre with isotonic saline.

1M Calcium chloride solution

$CaCl_2.2H_2O$ 14.7g

dH_2O 100mL

Tween wash buffer

10X TBS Stock Buffer 100mL

Nonidet P40 substitute 5mL

(Fluka BioChemika code 74385)

Tween 20 0.5mL

1M $CaCl_2$ 0.5mL

dH_2O make up to 1L Store at 4 ^oC

Add 1mL 20% BSA per 100mL before use.

Peroxidase conjugated Goat anti-human IgG (GAH:HRP)

Jackson ImmunoResearch (affinity purified/min. cross reactivity) (catalogue code; 109-035-098) Store at 4^{o}C

Reconstitute with water, dilute with equal volume Glycerol, aliquot (e.g. 20µL) and store at -20^{o}C. Before use dilute this stock solution to working concentration, e.g. 1 in 3,000 in Tween wash buffer (= 1/6000 final concentration). Discard un-used diluted reagent.

OPD (Dako S2045) Substrate Solution

OPD. 2HCl Store at 4^{o}C 4 tablets

dH_2O 12mL

30% H_20_2 Store at 4^{o}C. Add 5µL immediately before use.

Discard un-used reagent.

Note: To ensure operator safety, gloves must be worn when preparing and dispensing OPD.

Stop Solution 0.5M H_2SO_4

Store at room temp.

Platelet preparation

1. Take blood from group O donors into EDTA or citrate.

2. Centrifuge at 500g for 10 min in bench-top centrifuge

3. Remove the top 3/4 of the PRP from the top of the tube and transfer to 10mL conical centrifuge tube.

4. Make up to 10mL with PBS/EDTA buffer. Centrifuge at 2,000g for 5 mins and decant supernantant

5. Re-suspend cells gently in 2mL buffer and repeat step 4. twice more.

6. Re-suspend platelets in PBS/EDTA at 100x 10^{9}/L approx. Store at 4^{o}C for up to 2 weeks. Or if using cryopreserved platelets, recover using local method and resuspend at 100x 10^{9}/L.

Method

1. Preparation of Coated F-Well Microplate

1.1. Prepare goat anti-mouse IgG as above and aliquot 100mL/well into F-well microplates.

1.2. Attach a microplate sealer and incubate at 4^{o}C for at least 3 hours. Store sealed coated plates for up to 2 weeks at 4^{o}C.

2. Incubation of platelets with serum

2.1. Use panel platelets at 100x 10^{9}/L

2.2. Add 100µL platelets per well of a U-well plate according to the relevant plate plan.

2.3. Centrifuge microtitre plate 1400g for 3mins.

2.4. Discard supernatant and blot plate dry on paper towel.

2.5. Resuspend platelet pellet using a vortex mixer or plate shaker.

2.6. Add 50µL TBS/BSA buffer to each well.

2.7. Dispense 25µL test/control plasma to the wells of the plate according to the relevant plate plan.

2.8. Attach a microplate sealer to the plate if using a waterbath for incubation or a microtitre plate lid if using a dry air incubator.

2.9. Incubate at 37^{o}C for 30min in a waterbath or 40min in a dry air incubator.

3. Removal of unbound immunoglobulins

3.1. Centrifuge microtitre plate at 1400g for 3mins.

3.2. Discard supernatant and blot plate dry on paper towel.

3.3. Resuspend platelet pellet using a vortex mixer or plate shaker.

3.4. Add 200µL TBS/BSA to each well.

3.5. Attach plate sealer to microtitre plate.

3.6. Centrifuge microtitre plate at 1400g for 3mins.

3.7. Discard supernatant and blot plate dry on paper towel.

3.8. Resuspend platelet pellet using a vortex mixer or plate shaker.

3.9. Repeat steps 3.4 to 3.8 leaving the plate dry after the second and final wash (total of 2 washes).

4. Incubation of platelets with monoclonal antibody

4.1. Dilute monoclonal antibodies in TBS/BSA, as determined by prior experiment.

e.g. GPIIbIIIa: PAB-1 and PAB-6, 1 in 10;

GPIbIX: PAB-5, 1 in 10;

GPIaIIa: P16, 1 in 10;

β2 microglobulin: W6/32, 1 in 5;

CD109: 15E10, 1 in 100.

4.2. Add 50µL TBS/BSA buffer to each well.

4.3. Add 40µL of diluted Mab to the designated wells according to the relevant plate plan.

4.4. Attach plate sealer or plate lid as in 2.8

4.5. Incubate at 37^{o}C for 30min in a waterbath or 40min in a dry air incubator.

5. Block coated plates

5.1. Remove and discard capture antibody from plate prepared in step 1.

5.2. Add 125µL Tween wash buffer to all wells.

5.3. Remove and discard wash buffer, blotting excess on a paper towel.

5.4. Repeat steps 5.2 and 5.3 twice (total of 3 washes).

5.5. Add 125µL Tween wash buffer to all wells and leave for 30min at 22^{o}C/room temperature for use in step 8.1.

6. Removal of unbound monoclonal antibody

6.1. Centrifuge microtitre plate from step 4.5 at 1400g for 3mins.

6.2. Discard supernatant and blot plate dry on paper towel.

6.3. Resuspend platelet pellet using a vortex mixer or plate shaker.

6.4. Add 200µL TBS/BSA to each well.

6.5. Attach plate sealer to microtitre plate.

6.6. Centrifuge microtitre plate at 1400g for 3mins.

6.7. Discard supernatant and blot plate dry on paper towel.

6.8. Resuspend platelet pellet using a vortex mixer or plate shaker.

6.9. Repeat steps 6.4 to 6.8 twice, leaving the plate dry after the third and final wash (total of 3 washes).

7. Solubilisation of platelet membranes

7.1. Add 130µL of the solubilisation buffer to each well of the U-well plate from step 6.9 above and mix 3 times with a multichannel pipette.

7.2. Attach a plate sealer or plate lid as in 2.8.

7.3. Incubate at 22^{o}C for 15 minutes.

7.4. Centrifuge the plate at 1400g for 15 minutes to pellet cell stroma.

8. Transfer of platelet lysates to F-well plate

8.1. Take blocked F-well plate from step 5.5.

8.2. Remove and discard Tween wash buffer, blotting excess on a paper towel.

8.3. Transfer 100µL lysate supernatant from step 7.4 to corresponding wells of the coated, blocked F-well plate.

8.4. Attach plate sealer or plate lid as in 2.8.

8.5. Incubate plate at 37^{o}C for 30min in a waterbath or 40min in a dry air incubator.

9. Removal of unbound lysate proteins.

9.1. Discard supernatant and blot plate dry on paper towel.

9.2. Add 125µL of Tween wash buffer to each well.

9.3. Discard supernatant and blot plate dry on paper towel.

9.4. Repeat steps 9.2 and 9.3 five times (total of 6 washes).

10. Addition of peroxidase labelled Goat anti-human IgG

10.1. Prepare GAH:HRP as detailed in 'Reagents'.

10.2. Add 100µL diluted GAH:HRP to each well of the F-well plate.

10.3. Attach plate sealer or plate lid as in 2.8.

10.4. Incubate at 20^{o}C for 60 minutes.

11. Removal of unbound peroxidase labelled Goat anti-human IgG

11.1. Discard supernatant and blot plate dry on paper towel.

11.2. Add 125µL of Tween wash buffer to each well.

11.3. Discard supernatant and blot plate dry on paper towel.

11.4. Repeat steps 11.2 and 11.3 five times (total of 6 washes).

12. Addition of OPD substrate

12.1. Prepare OPD solution as detailed in 'Reagents' above.

12.2. Add 100µL OPD solution to all wells

12.3. Incubate at 22^{o}C in the dark for sufficient time to allow adequate colour development e.g. 10-20 minutes. Clear definition between positive and negative controls should be obtained.

13. Addition of acid to ‘stop’ colour development.

13.1. Add 100µL 0.5M H_2SO_4 to all wells.

14. Reading of microtitre plate

14.1. Read the plate in a microplate reader at 490nm using a suitable reference wavelength (630- 650nm).

14.2. Record OD’s after subtraction of reagent blank OD