

MACE® 1 (Modified Antigen Capture ELISA)

INTENDED USE

MACE® 1 is a qualitative solid phase enzyme linked immunosorbent assay (ELISA) designed to detect IgG antibodies to HLA class I antigens and to epitopes on the platelet glycoprotein IIb/IIIa.

For Research Use Only

SUMMARY AND EXPLANATION

The existence of platelet-specific antigens on various platelet glycoproteins has been described by many investigators.^{1,2,3,4,5,6} Antibodies to platelet-specific or HLA class I antigens due to pregnancy or transfusion can result in immune destruction of transfused platelets.^{7,8,9} Confirming the presence of these antibodies in patient sera can be helpful in the search for potentially compatible blood products.

MACE® 1 microwells provide immobilized monoclonal antibodies designed to capture HLA class I or platelet IIb/IIIa glycoproteins. The assay is designed for use in detecting and differentiating between HLA and platelet-specific antibodies directed against donor or patient platelets.

PRINCIPLE OF THE PROCEDURE

Patient serum or plasma is incubated with intact platelets allowing for antibody, if present, to bind to the platelet glycoproteins. Unbound antibodies are washed from the platelets. The antibody-sensitized platelets are solubilized by the addition of a lysis buffer containing a non-ionic detergent. The platelet lysate containing soluble glycoproteins is transferred to microwells. This allows the platelet and HLA class I glycoproteins (sensitized or unsensitized with patient antibody) to be captured by immobilized monoclonal antibodies. Control samples are handled similarly. After a brief incubation period, unbound glycoproteins are washed away. An alkaline phosphatase labeled anti-human globulin reagent, Anti-IgG, is added to the wells and incubated. The unbound Anti-IgG is washed away and the substrate PNPP (p-nitrophenyl phosphate) is added. After a 30-minute incubation period, the reaction is stopped by a sodium hydroxide solution. The optical density of the color that develops is measured in a spectrophotometer. A positive result indicates the presence of glycoprotein-specific antibody on the captured GPIIb/IIIa or HLA glycoproteins.

REAGENTS

Maximum number of tests per kit: 45

All reagents should be stored as directed by the label.

MS	1. Microwells: Flat-bottom microwell strips to which murine monoclonal antibodies specific for platelet and HLA class I glycoproteins have been immobilized. 6-1x8 RED strips specific for GPIIb/IIIa and 6-1x8 BLUE strips specific for HLA class I. The microwells are enclosed in a resealable foil pouch. Ready for use.
TCW	2. Concentrated Wash (10x): Tris (hydroxymethyl) ammoniummethane buffered solution containing sodium chloride and Tween 20. 1% sodium azide. Dilute with deionized or distilled water before use. Store Working Wash solution up to 48 hours at room temperature or up to seven days at 2 to 8°C.
TSD	3. Specimen Diluent: Tris buffered saline solution containing sodium chloride. 0.05% sodium azide. Ready for use.
SB	4. Substrate Buffer: This solution contains diethanolamine and magnesium chloride. 0.02% sodium azide. Ready for use. Protect from light.
SS	5. Stopping Solution: 3 M Sodium Hydroxide. Ready for use. Use with care.
AG	6. Conjugate: Alkaline phosphatase conjugated goat affinity purified antibody to human immunoglobulin G (IgG). 0.1% sodium azide. Dilute in Specimen Diluent before use.
PN	7. PNPP (p-nitrophenyl phosphate) Substrate: Crystalline powder. Reconstitute with deionized or distilled water and dilute in Substrate Buffer before use. Protect from light.
PC	8. Positive Serum Control: Human Serum. 0.1% sodium azide. Dilute in Specimen Diluent before use.
NC	9. Negative Serum Control: Human Serum. 0.1% sodium azide. Dilute in Specimen Diluent before use.
CRP	10. Cell Resuspension and Preservative Solution: Phosphate buffered saline solution, containing bovine albumin, 0.1% sodium azide. Ready for use.

CLB	11. Cell Lysis Buffer (10x): Tris buffered saline solution containing a non-ionic detergent. 0.1% sodium azide. Dilute with deionized or distilled water before use.
NCP	12. Normal Platelet Control: Dried pooled human platelets. Rehydrate before use with Cell Resuspension and Preservative Solution.
PS	13. Plate Sealers.

PRECAUTIONS

- Do not use reagents that are turbid or contaminated.
- Care MUST be taken to avoid contamination of Specimen Diluent and Conjugate. Inadvertent contamination of these reagents with human serum will result in the neutralization of the Conjugate and subsequently to test failure.
- Do not use reagents beyond their expiration date.
- Microwells and reagents contained in the kit are not to be used in conjunction with any other test system.
- Substitution of components other than those provided in this kit may lead to inconsistent or erroneous results.
- Discard any unused portions of diluted Conjugate, diluted Positive and Negative Controls, and diluted and reconstituted PNPP reagent after each run.
- Diluted Cell Lysis Buffer MUST be used on the day of preparation and not stored for future use.
- When making dilutions, follow pipet manufacturer's instructions for appropriate dispensing and rinsing techniques.
- The enzyme substrate reaction which occurs in the final incubation is temperature sensitive and should be performed in a controlled area at 22 to 25°C.
- Due to variations in instruments or consistently higher or lower room temperatures, it may be necessary for the laboratory to establish a slightly longer or shorter incubation time in order to consistently achieve valid control monitor. Because the temperature of the final incubation can affect control values, it is important to periodically monitor the room temperature incubation.

CAUTION

- All human serum used in the Positive and Negative Controls for this product has been tested and found negative for antibody to HIV, HCV, and HBsAg by FDA approved methods. No test method, however, can offer complete assurance that HIV, Hepatitis C virus, Hepatitis B virus or other infectious agents are absent. Therefore, these materials should be handled as potentially infectious.
- Some of the reagents supplied with this kit contain sodium azide as a preservative.
WARNING: Sodium azide reacts with lead and copper plumbing forming highly explosive metal azides. When discarded in a sink, the sink should be flushed with a large volume of water to prevent azide buildup. Sodium azide is a poison and is toxic if ingested.
- Stopping Solution (NaOH) is corrosive. Avoid contact with skin and eyes. Spills should be cleaned up immediately.
- Discard all components when completed according to local regulations.

SPECIMEN COLLECTION

Serum or Plasma:

Blood should be collected in ACD (plasma) or without anticoagulant (serum) using aseptic technique and should be tested while still fresh to minimize the chance of obtaining false positive or false negative reactions due to improper storage or contamination of the specimen.

Serum or plasma samples that cannot be tested immediately should be stored at 2 to 8°C for no longer than 48 hours or frozen. Samples frozen at -20°C or below remain in good condition for several years (2-5 years). However, in order to avoid the deleterious effect of repeated freeze/thaw cycles, it is recommended that samples should be aliquoted in small volumes and then stored frozen. Avoid frost-free freezers.

Serum or plasma should be separated from red cells when stored or shipped.

Only whole human serum or plasma is suitable for this assay. Prior dilution of samples in anything other than normal, ELISA negative human serum or plasma could affect the results.

Microbially contaminated, hemolyzed, lipemic or heat inactivated samples may give inconsistent test results and should be avoided.

Platelets:

- Platelets samples should be collected in CPD or ACDA and tested within 7 days. When not tested immediately, platelets should be stored at room temperature (20 to 25 °C). Platelet samples obtained from platelet concentrates should be used within prescribed dating period.
- 2 – 3 mL of platelet rich plasma (≤ 400,000 per µL) or 0.25 mL of platelet concentrate or 15 µL of a 50% suspension of washed platelets for each serum sample to be tested. Platelet-rich plasma can be prepared by centrifuging whole blood sufficiently to sediment red cells but leaving platelets in suspension.
- Platelets should be centrifuged at a time and speed adequate to pellet the cells. Excessive centrifugation, however, is not recommended. Suggested centrifugation times are 580 x g for 20 minutes, 2,000 x g for 10 minutes, or 5,000 x g for 6 minutes.
- Each laboratory should establish the optimal times and speeds for the equipment in use.

Platelet Storage:

- Prepare a platelet button as described in step 5 below. Discard the supernatant and resuspend the platelet button in 0.25 mL of Cell Resuspension and Preservative Solution. This suspension may be stored at 2 to 8°C for up to 7 days.

PROCEDURE

Materials Provided:

Vials may contain more reagent than described on the labels. Be sure to measure the reagent with an appropriate device when making dilutions.

1. 12 – 1 x 8 Microwell Strips with holder
2. 1 x 50 mL Concentrated Wash
3. 1 x 14 mL Specimen Diluent
4. 1 x 14 mL Substrate Buffer
5. 1 x 14 mL Stopping Solution
6. 1 x 80 : 1 Anti-Human IgG Conjugate
7. 6 x 50 mg PNPP Substrate
8. 1 x 0.7 mL Positive Serum Control
9. 1 x 0.7 mL Negative Serum Control
10. 3 Normal Platelet Controls (50 µL rehydrated)
11. 1 x 2.5 mL Cell Lysis Buffer
12. 1 x 50 mL Cell Resuspension and Preservative Solution
13. 12 Plate Sealers
14. 2 Recording Sheets

Additional Materials Required:

1. Test tubes for patient sample and control dilutions and for reagent dilutions
2. Transfer pipets
3. Adjustable micropipets to deliver 10 – 100 µL and 100 – 1,000 µL and disposable tips
4. Timer
5. Microplate reader capable of measuring OD at 405 or 410 and 490 nm
6. Deionized or distilled water
7. Absorbent paper towels
8. Microplate washer or device
9. Centrifuge capable of separating serum or plasma from platelets
10. 37°C waterbath or incubator
11. Microcentrifuge tubes
12. Microcentrifuge for pelleting platelets

Test Procedure

1. Bring all reagents to room temperature.
2. Make Working Wash solution by diluting Concentrated Wash. Add 1 volume of Concentrated Wash to 9 volumes of deionized or distilled water. Mix well.
3. Determine the number of patient samples to be tested. Using the Recording Sheet, assign each sample to a location consisting of one well on each strip. Record the identity of each sample on the Recording Sheet.

PREPARE SAMPLES AND CONTROLS

4. Prepare the positive and negative controls:
 - a) Rehydrate one vial of the dried Normal Platelet Control by adding 400 : 1 of Cell Resuspension and Preservative Solution.

- b) Allow to stand for 10 to 30 minutes at room temperature.
- c) Mix the rehydrated platelets well.
- d) Centrifuge tubes to pellet cells.
- e) Decant supernatant and blot the tubes dry.
- f) Add 50 µL of Cell Resuspension and Preservative Solution to the platelet button.
- g) Mix well with the aid of a pipet tip to obtain a homogeneous suspension. Transfer 15 µL of the platelet suspension into each of two clean microcentrifuge tubes.
- b) Add 150 : L of Positive Serum Control to one of the tubes. Label as positive control. Add 150 : L of Negative Serum Control to the other tube. Label as negative control. MIX WELL.

Prepare test platelets:

5. For each platelet sample to be tested, place 2 – 3 mL of platelet rich plasma (platelet count should not exceed 400,000 per : L) or approximately 0.25 mL platelet concentrate in a microcentrifuge tube and centrifuge to obtain a platelet button. Discard supernatant plasma.

6. Add 400 : L of Cell Resuspension and Preservative Solution to the platelet button and MIX WELL. Centrifuge to obtain a platelet button.

7. Invert the tube and discard the supernatant fluid. Repeat steps 6 and 7 for a total of 3 to 4 washes. Following the last wash, blot tubes with an absorbent paper towel. Allow the supernatant to be drained away.

8. Estimate the volume of each platelet button. Make a 50% suspension of each platelet by adding an equal volume of Cell Resuspension and Preservative Solution to the platelet button. Mix well to obtain a homogeneous suspension. Transfer 15 µL of the 50% platelet suspension to a clean labeled tube.

9. Add 150 : L of patient serum or plasma to each of the tubes containing a platelet test suspension prepared in the previous step. MIX WELL with the aid of a pipet.

10. Incubate the positive and negative control and test samples for 30 – 35 minutes at 37°C. If a dry incubator is used, increase the incubation time by 10 minutes.

11. Wash all test and control samples twice by adding 400 : L of Cell Resuspension and Preservative Solution to each tube and centrifuging to pellet the platelets. Discard supernatant fluid after each wash. Blot tubes with absorbent paper to remove all residual fluids, being careful not to disturb the platelet button after final wash.

12. Dilute Cell Lysis Buffer. Add 100 µL of Cell Lysis Buffer to 900 µL of deionized or distilled water. Prepare 1.0 mL diluted buffer for each five platelets to be lysed. MIX WELL.

13. To lyse the platelets, add 180 : L of diluted Cell Lysis Buffer (prepared in the previous step) to each test and control tube. MIX WELL with the aid of a pipet or vortex to obtain complete lysis. Promptly proceed to the assay. Platelet lysates should be tested as soon as possible.

PERFORMING THE ASSAY

14. Remove microwell frame from pouch. Promptly remove and reseal unneeded strips in the protective pouch.

NOTE: Only one frame is provided in the kit. Do not discard until all strips have been used. The strips, when placed in the frame, should have the colored mark(s) at the top. Be sure that the order of the strips in the frame matches the order on the Recording Sheet.

15. Add 50 µL of sensitized platelet lysate, negative control and positive control lysate to the wells designated on the Recording Sheet.

NOTE: Do not add samples or reagents to blank wells.

NOTE: LABEL EACH STRIP TO AVOID ERRORS.

16. Seal the microwells with a plate sealer and incubate for 30 – 35 minutes in a 37°C waterbath. If a dry incubator is used instead, increase time by 10 minutes.

17. Dilute the Conjugate 1 to 100 in Specimen Diluent. Use a polypropylene container.

Strip:	2 – 1 x 8	12 – 1 x 8
IgG	10 µL	60 µL
TSD	1.0 mL	6.0 mL

NOTE : Conjugate is viscous. Prime tip 2 – 3 times in Conjugate before dispensing and rinse after addition to Specimen Diluent. Mix well.

18. WASH STEP:

- a) Aspirate or decant contents of each well and blot on absorbent toweling.
- b) Add 300 µL Working Wash solution.
- c) Aspirate or decant.
- d) Repeat steps b + c for a total of 3 or 4 washes.
- e) Vigorously decant to remove all residual wash solution. Invert on absorbent toweling to prevent drying.

NOTE: It is important to completely remove all wash solution after the final wash.

19. Add 50 µL of diluted Conjugate (made in a previous step) to all wells EXCEPT those designated as BLANKS.

20. Seal the microwells with a plate sealer and incubate for 30 – 35 minutes in a 37°C waterbath. If a dry incubator is used, increase time by 10 minutes.

21. Dissolve PNPP Substrate by adding in 0.5 mL deionized or distilled water to the vial. Replace stopper and mix well. Protect from light until use.

22. Dilute the PNPP 1 to 100 in the Substrate Buffer.

Strip:	2 – 1 x 8	12 – 1 x 8
PN	20 µL	120 µL
SB	2.0 mL	12.0 mL

Mix well. Protect from light until use.

23. WASH STEP:

- a) Aspirate or decant contents of each well and blot on absorbent toweling.
- b) Add 300 µL Working Wash solution.
- c) Aspirate or decant.
- d) Repeat steps b + c for a total of 3 or 4 washes.
- e) Vigorously decant to remove all residual wash solution. Invert on absorbent toweling to prevent drying.

Proceed quickly through next three steps.

24. Add 100 µL of the diluted PNPP solution to all the wells EXCEPT those designated as BLANKS.

25. Allow the microwells to stand in the dark for 30 minutes at ROOM TEMPERATURE (22 to 25°C).

NOTE: Incubation time and temperature after the addition of PNPP is critical. DO NOT vary the established incubation time or temperature. For consistency, begin timing promptly after addition of the reagent to the first well.

26. Stop the reaction by adding 100 µL of Stopping Solution to each well in the same sequence as the addition of substrate. Add 200 µL of Stopping Solution to the blank wells.

27. Read the absorbance (OD) of each well at 405 or 410 nm using a reference filter of 490 nm. If the results cannot be read immediately, return the wells to a dark location for up to 30 minutes.

28. Subtract the values obtained in the blank wells from all sample and control wells. Many ELISA readers are programmed to automatically perform this step.

29. Record the results on the Recording Sheet.

QUALITY CONTROL

Quality control of MACE®¹ is built into the test system by the inclusion of Positive and Negative Controls. These controls should be included with each test run to help determine if technical errors or reagent failures have occurred.

Criteria for a valid test:

OD	Negative Control ≤ 0.150 (HLA row)	Positive Control ≥ 1.800 (HLA row)
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INTERPRETATION OF TEST RESULTS

Test results showing OD values equal to or greater than 2X the value obtained for the negative control platelet of corresponding glycoprotein are regarded as positive results.

LIMITATIONS

Erroneous results can occur from bacterial contamination of test materials, inadequate incubation periods, inadequate washing or decanting of test wells or of the test platelets, exposure of substrate to stray light, omission of test reagents, exposure to higher or lower than prescribed temperature requirements, insufficient or excessive platelets, incompatible ABO groups, or omission of steps.

The results of this assay should not be used as the sole basis for a clinical decision.

Some low titer, low avidity antibodies may not be detected using this assay.

This product does not detect IgM or IgA antibodies, or antibodies to platelet glycoproteins other than those on Ib/IIIa and HLA Class I.

In vivo sensitized platelets have not been tested using this product.

REFERENCES

1. Kunicki TJ, Aster RH: Isolation and immunologic characterization of the human platelet isoantigen P1(A1). Mol Immunol 1979; **16**: 353
2. Friedman JM, Aster RH: Neonatal alloimmune thrombocytopenic purpura and congenital porcephaly in two siblings associated with a new maternal antiplatelet antibody. Blood 1985; **65**: 1412
3. Furihata K, Nugent DJ, Aster RH, Kunicki TJ: Anti-Pe1(a) binds specifically to an epitope on platelet glycoprotein IIb. Blood 1986; **68**: 107 (suppl 1) (abstr)
4. Simon T, Collins J, Kunicki T, Furihata K, Smith K, Aster RH: Post-transfusion purpura with antiplatelet antibody specific for the platelet antigen Pe1. Blood 1986; **68**: 117 (abstract)
5. Woods VL, Oh EH, Mason D, McMillan R: Autoantibodies against the platelet glycoprotein Ib/IIIa complex in patients with chronic ITP. Blood 1984; **63**: 368
6. McMillan R, et al. Blood 1987; **70**: 1040-1045
7. Howard JE, Perkins HA: The natural history of alloimmunization to platelets. Transfusion 1978; **18**: 496
8. Dutcher JP, Schiffer CA, Aisner J, Wiemik PH: Alloimmunization following platelet transfusion: the absence of dose-response relationship. Blood 1981; **57**: 395
9. Schiffer CA: Clinical importance of antiplatelet antibody testing for the blood bank. In: A seminar on antigens on blood cells and body fluids. Washington DC: American Association of Blood Banks; 1980: 189-208



MACE®¹

- FOR RESEARCH USE ONLY
- STORE AT 2 to 8°C

20925 Crossroads Circle, Suite 200
Waukesha, WI 53186-4054 USA
(262) 754-1000 OR 1-800-233-1843

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www.gtdiagnostics.com