

INTENDED USE

PAKAUTO® is a qualitative solid phase enzyme linked immunosorbent assay (ELISA) designed to detect platelet autoantibodies eluted from patient’s platelets or circulating in the patient’s serum or plasma.

For *In Vitro* Diagnostic Use.

SUMMARY OF EXPLANATION

Autoimmune thrombocytopenic purpura (AITP) is one of the most common causes of immune thrombocytopenia. About half of AITP cases occur in association with other conditions such as lymphoma, systemic lupus erythematosus (SLE) and HIV infection; the remaining cases are considered to be “idiopathic.” On the basis of clinical presentation, AITP can be classified into two types: acute AITP, a childhood disorder which is usually self-limited, and chronic AITP occurring in adults which rarely remits spontaneously.

Most antibodies associated with AITP recognize platelet membrane glycoproteins, especially GPIIb/IIIa, GPIb/IX, and GPIa/IIa.^{1,2} Antibodies reactive with these targets can often be detected in plasma of patients with AITP,^{1,2,3,4,5} but it is preferable to characterize platelet-associated immunoglobulins to confirm auto-reactivity. Many tests for identification of platelet-associated immunoglobulins are lacking in specificity in that they often yield positive results in patients with non-immune types of thrombocytopenia.²

PAKAUTO® Solid Phase ELISA microwells contain monoclonal-captured glycoproteins IIb/IIIa, Ib/IX, and Ia/IIa. The assay is designed to detect platelet glycoprotein-specific autoantibodies in patient serum or plasma, or eluted from the surface of their platelets.

PRINCIPLE OF THE PROCEDURE

Patient serum, plasma or eluate prepared from patient platelets is added to microwells coated with platelet glycoproteins allowing antibody, if present, to bind. Unbound antibodies are then washed away. An alkaline phosphatase labeled anti-human globulin reagent (Anti-IgG/A/M) is added to the wells and incubated. The unbound Anti-IgG/A/M 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.

REAGENTS

Maximum number of tests per kit: 5
All reagents should be stored as directed by the label.

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|------------|--|
| MS | 1. Microwells: Flat-bottom microwell strips to which platelet glycoproteins IIb/IIIa, Ib/IX, and Ia/IIa, have been immobilized. The microwells are enclosed in a resealable foil pouch. Ready for use. |
| TCW | 2. Concentrated Wash (10x): Tris (hydroxymethyl) aminomethane 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. |
| SD | 3. Specimen Diluent: Phosphate buffered saline solution containing bovine albumin and mouse serum. 0.1% 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. |
| AH | 6. Conjugate: Alkaline phosphatase conjugated goat affinity purified antibody to human immunoglobulins (IgG/A/M). 0.1% sodium azide. Dilute in Specimen Diluent before use. |

- | | |
|------------|--|
| CRP | 7. Cell Resuspension and Preservative Solution: Phosphate buffered saline solution containing EDTA. 0.1% sodium azide. Ready for use. |
| BS | 8. Buffering Solution: Tris solution containing bovine albumin. 0.1% sodium azide. |
| ES | 9. Eluting Solution: A low pH glycine buffer. |
| PN | 10. PNPP (p-nitrophenyl phosphate) Substrate: Crystalline powder. Reconstitute with deionized or distilled water and dilute in Substrate Buffer before use. Protect from light. |
| PC | 11. Positive Serum Control: Human Serum. 0.1% sodium azide. Dilute in Specimen Diluent before use. |
| NC | 12. Negative Serum Control: Human Serum. 0.1% sodium azide. Dilute in Specimen Diluent before use. |
| PCP | 13. Positive Platelet Control (Positive Eluate Control): Vacuum dried human platelets coated with antibody. Rehydrate before use with Cell Resuspension and Preservative Solution. |
| NCP | 14. Normal Platelet Control (Negative Eluate Control): Vacuum dried pooled human platelets. Rehydrate before use with Cell Resuspension and Preservative Solution. |
| PS | 15. 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 or plasma 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.
- When making dilutions, follow pipet manufacturer's instructions for appropriate dispensing and rinsing techniques.
- The enzyme substrate reaction occurring 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 results. 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 have 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

Blood should be collected in EDTA (plasma, platelets) 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 (2-3 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.

Particulates or aggregates in the sample can cause false positive results or poor duplicate values. Samples containing particulate matter should be clarified by centrifugation prior to testing.

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

For preparation of platelet eluates, a minimum of 1×10^7 platelets are required (5×10^7 platelets are preferred). Sufficient sample can be obtained from patients with platelet counts of $\geq 10,000/\mu\text{L}$ by collecting two 7 mL tubes of whole blood.

NOTE: Shipment of blood samples can result in reduced platelets. When samples are to be transported, it is better to collect a larger sample to assure adequate platelets for elution.

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. 6 – 2 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 μL Anti-Human IgG/A/M Conjugate
7. 1 x 50 mL Cell Resuspension and Preservative Solution
8. 1 x 2.5 mL Buffering Solution
9. 1 x 2.5 mL Eluting Solution
10. 3 x 50 mg PNPP Substrate
11. 1 x 0.3 mL Positive Serum Control
12. 1 x 0.7 mL Negative Serum Control
13. 1 Positive Platelet Control (Positive Eluate Control)
14. 1 Normal Platelet Control (Negative Eluate Control)
15. 6 Plate Sealers

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 patient samples
10. 37°C waterbath or incubator
11. Microcentrifuge tubes

12. Microcentrifuge for pelleting platelets
13. Normal platelets (5×10^7) for elution control

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, eluate or serum/plasma to be tested. Using the Recording Sheet, assign each sample to a location consisting of one column. Record the identity of each sample on the Recording Sheet.

PREPARE SAMPLES AND CONTROLS

4. Dilute as follows and mix well:

	Volume Specimen Diluent	Volume Sample
PC	150 μL	50 μL
NC	300 μL	100 μL
Patient serum or plasma	300 μL	100 μL

5. Prepare platelet pellets from patient or normal donor platelets as follows:
 - a) Prepare platelet rich plasma (PRP) by centrifuging EDTA blood samples at 110 –150 rcf for 10 - 15 minutes.
 - b) Transfer PRP to a clean polypropylene test tube and centrifuge at 10,600 rcf for 5 - 10 minutes to obtain a platelet button. Remove plasma.
 - c) Add 500 μL of CRP to the platelet pellet and mix gently with a small bore pipet. Transfer the platelet suspension to a microfuge tube and wash three times with at least 500 μL of Cell Resuspension and Preservative Solution (CRP).
 - d) After the final wash, prepare a platelet pellet containing between 1 and 5×10^7 platelets. This can be done by resuspending the platelets with CRP and adjusting the platelet count to 10,000 – 50,000 platelets per μL . Transfer 1 mL to a microfuge tube and centrifuge for 5 min at 10,600 rcf to pellet. (Alternatively, a 5 - 10 μL pellet of platelets should contain enough platelets to perform the elution. Compare the pellet to an identical tube containing 5 - 10 μL of Specimen Diluent.)
6. Prepare control platelet pellets:
 - a) Add 500 μL of Cell Resuspension and Preservative Solution to the Positive Platelet Control (Positive Eluate Control) and, if needed, the Normal Platelet Control (Negative Eluate Control). Allow to stand at room temperature for at least 10 minutes to re-hydrate. Mix gently to resuspend and centrifuge at 10,600 rcf for 5 - 10 minutes. Decant or aspirate supernatant and blot remaining buffer to obtain a dry button of platelets.

NOTE: It is preferable to use fresh normal platelets for the Negative Eluate Control but a dried platelet has been provided for occasions when normal platelets are not available.

7. Prepare the eluates:
 - a) Before proceeding, make sure that all platelet pellets are well sedimented and all remaining CRP is removed.
 - b) Add 180 μL of Elution Solution to each platelet button and mix with the aid of a pipet. Allow the mixture to stand at room temperature for 2 minutes. Centrifuge at 10,600 rcf for 5 - 10 minutes in a microfuge tube.
 - c) Promptly transfer the supernatants (eluates) to a clean microfuge tube and add 180 μL of Buffering Solution to each tube. Mix thoroughly. Centrifuge at 10,600 rcf for 10 minutes in a microfuge tube to remove any platelet debris.
8. Test promptly or freeze for future testing. Platelet eluates can be stored frozen at -80°C for use in future testing.

NOTE: Do not dilute eluates.

9. 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.

NOTE: Orient the frame with A1 in the top left corner. Be sure that all strips are properly seated and snapped into their frame. Label or number each strip to avoid errors. Maintain the same plate orientation throughout the assay.

10. Add 300 μ L of Working Wash solution to all wells and allow to stand at room temperature for 5-10 minutes.
11. Aspirate or decant forcefully and invert on absorbent toweling to prevent drying.
12. Add 50 μ L of the appropriate eluate or diluted control or sample to the wells of one column shown in Figure 1.

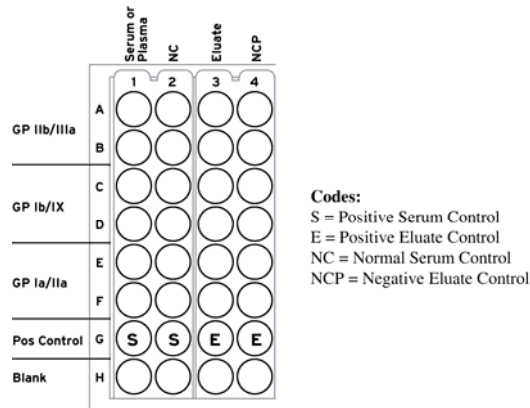


Figure 1

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

NOTE: If multiple patient samples are tested at the same time, only one set of controls is required. LABEL EACH STRIP TO AVOID ERRORS.

12. 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.
14. Dilute the Conjugate 1 to 100 in Specimen Diluent. Use a polypropylene container.

Strips:	2 – 2 x 8	6 – 2 x 8
AH	20 μ L	60 μ L
SD	2.0 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.

15. WASH STEP:

- a) Aspirate or decant contents of each well on absorbent toweling.
- b) Add 300 μ L 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.

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

17. 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.

18. Dissolve PNPP Substrate by adding 0.5 mL deionized or distilled water to the vial. Replace the stopper and mix well. Protect from light until use.
19. Dilute the PNPP 1 to 100 in the Substrate Buffer.

Strips:	2 – 2 x 8	6 – 2 x 8
PN	40 µL	120 µL
SB	4.0 mL	12.0 mL

Mix well. Protect from light until use.

20. WASH STEP:

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

Proceed promptly through next three steps.

21. Add 100 µL of the diluted PNPP solution to all the wells EXCEPT those designated as BLANKS.
22. 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.

23. Stop the reaction 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.
24. 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.
25. Subtract the values obtained in the blank wells from all sample and control wells. Many ELISA readers are programmed to automatically perform this step.
26. Record the results on the Recording Sheet.

QUALITY CONTROL

Quality control of PAKAUTO[®] 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:

	Negative Control	Positive Control
Eluate	≤ 0.100 (IIb/IIIa row)	≥ 1.000
Serum	≤ 0.160 (IIb/IIIa row)	≥ 1.000

OD readings obtained for duplicate test results should fall within 20% of the mean of the two values. Samples whose results are outside of this limit should be re-tested.

NOTE: Poor duplicates can be the result of reagent or sample omission, uneven addition of reagents, uneven temperature during incubations, stray light during the final incubation or cross-well contamination. Failure to test in duplicate may lead to acceptance of erroneous results.

INTERPRETATION OF TEST RESULTS

Test results showing OD values equal to or greater than 2X the value obtained for the mean of the negative controls of the corresponding glycoprotein (2 negative control values for each 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, exposure of substrate to stray light, omission of test reagents, exposure to higher or lower than prescribed temperature requirements, insufficient or excessive platelets, or omission of steps.

The presence of immune complexes or other immunoglobulin aggregates in the patient sample may cause an increased non-specific binding and produce false-positives in this assay.

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 is specifically designed to detect autoantibodies eluted from patient's platelets. Therefore, a positive reaction obtained only with patient serum or plasma does not necessarily indicate the presence of an autoantibody. Platelet-specific alloantibodies may also be reactive using this assay.

PAKAUTO[®] is designed to detect autoantibodies reactive with platelet glycoproteins IIb/IIIa, Ib/IX, and Ia/IIa. Autoantibodies to other platelet proteins are not expected to react in this assay.

It is possible that an autoantibody could give a false negative result in this assay due to steric hindrance of the human autoantibodies by the murine monoclonal antibodies used to capture the platelet glycoprotein.

SPECIFIC PERFORMANCE CHARACTERISTICS

When properly stored and used according to the procedures described above, this product can detect autoantibodies to platelet glycoproteins.

To ensure suitable reactivity and specificity, each lot of PAKAUTO[®] is tested prior to release with samples known to contain alloantibodies or autoantibodies reactive with the glycoproteins identified on the enclosed Recording Sheet as well as samples known to be free of such antibodies.

Performance Evaluation

		Comparative Method		
		Positive	Negative	Total
PAKAUTO [®]	Positive	24	1	25
	Negative	23	49	72
	Total	47	50	97

Agreement: 75.3%

Co-positivity: 51.1% Co-negativity: 98%

Comparative Method: Platelet Associated IgG* (Flow Cytometry)
*PAIgG has a low positive predictive value for ITP.^{6,7}

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PAKAUTO®

- FOR *IN VITRO* DIAGNOSTIC USE
- STORE AT 2 to 8°C

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