Beaumont

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Heparin Platelet Factor 4 IgG Assay

Document Type: Procedure .

I. PURPOSE AND OBJECTIVE:

To describe how to perform Heparin Platelet Factor 4 IgG Assay tests.

II. PRINCIPLE:

The Heparin PF4 IgG test is a qualitative screening assay used for the detection of IgG antibodies that are directed against a heparin-platelet factor 4 complex. Patient sample is added to micro-wells coated with platelet factor 4 (PF4) complexed to polyvinyl sulfonate (PVS). If an antibody recognizing a site on PF4: PVS is present, binding will occur. Unbound antibodies are then washed away. An alkaline phosphatase labeled anti-human globulin (Anti-IgG) reagent is added to the wells and incubated. The unbound Anti-IgG is washed away and the substrate p-nitrophenyl phosphate (PNPP) is added. After a 30 minute incubation period, the reaction is stopped with stopping solution. The optical density of the color that develops is measured by spectrophotometry.

III. CLINICAL SIGNIFICANCE:

Patients receiving heparin treatment for at least a week may develop thrombocytopenia. In some cases, the platelet levels are reduced only slightly and return to normal even when heparin treatment is continued. This type of thrombocytopenia is termed “Type I” heparin-induced thrombocytopenia (HIT) and is not antibody-mediated. In other patients, thrombocytopenia is more severe and is antibody-mediated. This condition is designated “Type II” HIT. Type I HIT is generally considered to be a benign condition, whereas patients with Type II HIT are at risk of developing more severe thrombocytopenia as well as arterial or venous thrombosis if heparin therapy is continued. Antibodies associated with Type II HIT can be detected in several ways. The most commonly used techniques are the platelet aggregation test, the serotonin release test[5] and the platelet factor 4 ELISA. It is now known that antibodies associated with Type II HIT recognize sites on a platelet protein designated “platelet factor 4” (PF4) that are created when PF4 is complexed with heparin or another linear polyanionic compound such as polyvinyl sulfonate (PVS). PF4 IgG Solid Phase ELISA microwells provide immobilized PF4: PVS complexes as a target for the detection of IgG antibodies associated with Type II HIT. Details can be found on the Beaumont intra-website by navigating to References>All References>Anticoagulation Resources>HIT (Heparin-Induced Thrombocytopenia) Management Algorithm. As part of the algorithm, patient samples with an absorbance (OD) between 0.4 and 1.999 will be sent out for

serotonin release assay testing. See the section of this procedure regarding the Send-Out Protocol.

IV. SPECIMEN COLLECTION AND HANDLING:

A. Blood should be collected without anticoagulant (serum) in a red-top tube using aseptic technique and should be tested while still fresh to minimize the change of obtaining false positive or false negative reactions due to improper storage or contamination of the specimen. Samples collected in serum separator tubes (SST) are not acceptable. Samples that cannot be tested immediately should be stored at 2-8°C for no longer than 48 hours or frozen. Samples frozen at -20°C or below remain in good condition for up to 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.

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

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

D. Hemolyzed, lipemic, or icteric samples may give inconsistent test results and should be avoided.

E. **WARNING:** Samples anti-coagulated with heparin should not be used in this assay.

F. Specimens received in the laboratory after 10:00 am will be tested on the following day.

NOTE: If the quantity is not sufficient to complete confirmation testing, cancel test and call for recollection.

V. PRECAUTIONS:

A. Do not use reagents that are turbid or contaminated.

B. 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.

C. Do not use reagents beyond their expiration date.

D. Micro-wells and reagents contained in the kit are not to be used in conjunction with any other test system.

E. Substitution of components other than those provided in this kit may lead to inconsistent or erroneous results.

F. Discard any unused portions of diluted conjugate, diluted positive and negative controls and diluted and reconstituted PNPP reagent after each run.

G. When making dilutions, follow pipette manufacturer's instructions for appropriate dispensing and rinsing techniques.

H. The enzyme substrate reaction which occurs in the final incubation is temperature sensitive and should be performed in a controlled area at 22-25°C.

I. Due to variations in instruments or consistently higher or lower room temperatures, it is recommended that the room temperature be monitored periodically.

J. All human serum used in the positive and negative controls for this product has been tested and found negative for antibody to human immunodeficiency virus (HIV), Hepatitis C virus (HCV), and Hepatitis B virus (HBsAg) by the Food and Drug Administration (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.

K. Some of the reagents supplied with this kit contain sodium azide as a preservative. 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 build-up. Sodium azide is a poison and is toxic if ingested.

L. Discard all components when completed by following local regulations.

VI. REAGENTS:

A. **Materials Provided**

Each kit contains the following components in sufficient quantities to perform the number of tests indicated on the packaging label. Vials may contain more reagent than described on the labels. Be sure to measure the reagent with an appropriate device when making dilutions.

NOTE: Some reagents contain sodium azide as a preservative at a concentration of 0.1% (w/v).

1. Micro-well Strips (12): 8 well flat-bottom micro-well strips to which affinity purified platelet factor 4 (PF4) complexed to polyvinyl sulfonate (PVS), has been immobilized. The micro-wells are enclosed in a re-sealable foil pouch. Ready for use.
2. PF4 Concentrated Wash (10x) (50 mL): 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 7 days at 2-8°C.
3. Specimen Diluent (30 mL): Phosphate buffered saline solution. 0.05% sodium azide. Ready for use.
4. Substrate Buffer (14 mL): This solution contains diethanolamine and magnesium chloride. 0.02% sodium azide. Ready for use. Protect from light.
5. Stopping Solution (14 mL): Ready for use.
6. Anti-Human IgG Conjugate (80 mcL): Alkaline phosphatase conjugated goat affinity purified antibody to human immunoglobulin (IgG). 0.1% sodium azide. Dilute in specimen diluent before use.
7. PNPP Substrate (6 x 50 mg): (p-nitrophenyl phosphate) Crystalline powder. Reconstitute with deionized or distilled water and dilute in substrate buffer before use. Protect from light.
8. Positive Serum Control (100 mcL): Human serum containing bovine albumin. 0.1% sodium azide. Dilute specimen in diluent before use.
9. Negative Serum Control (100 mcL): Human serum. 0.1% sodium azide. Dilute in specimen diluent before use.
10. Plate Sealers.

B. **Equipment/Supplies Required but Not Provided**

1. Polypropylene test tubes for patient sample and control dilutions and for reagent dilutions
2. Transfer pipettes
3. Eppendorf adjustable micropipette to deliver 294 L
4. Adjustable and repeater micropipettes to deliver 1-10 mcL, 10-100 mcL, and 100-1,000 mcL
5. Disposable micropipette tips
6. Timer

7. Microplate reader capable of measuring OD at 405 or 410 and 490 nm
8. Deionized or distilled water
9. Absorbent paper towels
10. Microplate washer
11. Centrifuge
12. 30°C water bath or incubator
13. Heparin, Porcine, USP 5,000 U/mL

C. **Storage Requirements**
Store the unopened kit between 2° and 8°C.

NOTE: Coated microwell strips: Store between 2° and 8°C. Extra strips should be immediately resealed and remaining components should be returned to proper storage.

VII. QUALITY CONTROL:

Quality control of PF4 IgG is built into the test system by the inclusion of positive and negative serum controls. These controls must be included with each test run to help determine if technical errors or reagent failures have occurred.

The criterion for a valid test follows:

	Negative Control	Positive Control
Mean Optical Density (OD)	≤0.300	≥1.800

OD readings obtained from duplicate tests must fall within 20% of the mean of the two values. Samples whose results are outside this limit should be retested.
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.
When the confirmation procedure is performed on positive samples, the positive control must be inhibited by >50% in the presence of excess heparin.

VIII. PROCEDURE:

A. Bring all reagents to room temperature.

B. Make working wash solution by diluting PF4 concentrated wash (10x) 1:10 with deionized or distilled water. Add bottle of concentrated wash (50 mL) to 450 mL of deionized or distilled water. Mix well.

C. Determine the number of patient samples to be tested. Two 1 x 8 micro-well strips are sufficient to perform five samples in duplicate. Using the pending log, assign each sample a number and a location consisting of two (duplicate) wells. The strips, when placed in the frame, must be oriented as shown in the diagram below. The notched end must be at the top.

	1

Blank A	**O**
Blank B	**O**
Positive Control C	**O**
Positive Control D	**O**
Negative Control E	**O**
Negative Control F	**O**
Patient 1 G	**O**
Patient 1 H	**O**

D. Dilute as follows and mix well:

	Volume of Specimen Diluent	Volume of Sample
Positive Control	294 mcL	6 mcL
Negative Control	294 mcL	6 mcL
Patient Sample(s)	294 mcL	6 mcL

NOTE: Precise measurement of patient and control samples are essential for accurate results.

E. Remove micro-well frame from pouch. Promptly remove and reseal unneeded strips in the protective pouch. Only one frame is provided in the kit. Do not discard until all strips have been used. Orient the frame with A1 in the top left corner. Be sure that all strips are properly seated and snapped into the frame. Label or number each strip to avoid errors. Maintain the same plate orientation throughout the assay.

F. Using an Eppendorf manual repeater pipette, add 300 mcL of working wash solution to all wells and allow to stand at room temperature for 5-10 minutes.

G. Aspirate or decant forcefully and invert on absorbent toweling to prevent drying.

H. Add 50 mcL of the appropriate diluted control or sample to the wells as designated on the pending log (positive control: C1 and D1, negative control: E1 and F1, patient 1 G1 and H1, patient 2 A2 and B2, patient 3 C2 and D2, etc.). Do not add samples or reagents to the blank wells (A1 and B1).

I. Seal the micro-wells with a plate sealer and incubate for 30-35 minutes in a 37°C water bath.

J. Dilute the conjugate 1 to 100 in specimen diluent. Use a polypropylene container.

Strips	**1 or 2**	**4**	**12**
Conjugate	10 mcL	20 mcL	60 mcL
Diluent	1.0 mL	2.0 mL	6.0 mL

NOTE: Conjugate is viscous. Prime the pipette tip 2-3 times in conjugate before dispensing and rinse after addition of specimen diluent. Mix well.

K. Follow the wash steps below:

1. Aspirate or decant the contents of each well and blot on absorbent toweling.
2. Add 300 mcL working wash solution.
3. Aspirate or decant.

4. Repeat steps b and c for a total of 3 washes.
5. Vigorously decant to remove all residual wash solution. Invert on absorbent toweling to prevent drying. It is important to remove all wash solution after the final wash.

L. Using an Eppendorf manual repeater pipette, add 50 mcL of diluted conjugate (made in previous step) to all wells except those designated as blanks (A1 and B1).

M. Seal the micro-wells with a plate sealer and incubate for 30-35 minutes in a 37°C water bath.

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

O. Follow the wash steps from item 11. Then proceed promptly through the next three steps.

P. Dilute the PNPP 1 to 100 in the substrate buffer.

Strips	**1 or 2**	**4**	**12**
PNPP	20 mcL	40 mcL	120 mcL
Substrate Buffer	2.0 mL	4.0 mL	12.0 mL

Mix thoroughly. Keep away from direct light. This reagent should be used immediately after preparation.

Q. Using an Eppendorf manual repeater pipette, add 100 mcL of the diluted PNPP solution to all the wells except those designated as blanks (A1 and B1).

R. Place the plate inside the appropriately labeled drawer and allow the microwells to stand in the dark for 30 minutes at room temperature (22-25°C). 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 the addition of the reagent to the first well.

S. Using an Eppendorf manual repeater pipette, stop the reaction by adding 100 uL of stopping solution to each well in the same sequence as the addition of substrate. Add 200 uL of stopping solution to the blank wells (A1 and B1).

T. Read the OD of each well at 405 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.

U. OD readings obtained from duplicate test results should fall within 20% of the mean of the two values. Subtract the values obtained in the blank wells from all sample and control wells. The ELISA readers are programmed to automatically perform this step.

V. Record the results on the pending log in the spaces provided.

IX. PROCEDURE FOR CONFIRMATION OF HEPARIN ASSOCIATED ANTIBODIES:

A. Add 20 mcL of heparin (5,000 U/mL) to 1 mL of specimen diluent to yield a final concentration of 100 units/mL.

B. Following the steps above, dilute the patient(s), positive and negative controls in the specimen diluent solution provided in the kit (see step 4 from procedure above). Additionally, dilute the patient(s) and positive control with the heparin/specimen diluent solution that was prepared in step 1 in the same manner.

C. Assign each sample a number and a location consisting of two (duplicate) wells. The strips, when placed

in the frame, must be oriented as shown in the diagram below. The notched end must be at the top.

	1		2
Blank A	**O**	**Patient 1 (with Heparin) A**	**O**
Blank B	**O**	**Patient 1 (with Heparin) B**	**O**
Negative Control (without Heparin) C	**O**	**Patient 1 (without Heparin) C**	**O**
Negative Control (without Heparin) D	**O**	**Patient 1 (without Heparin) D**	**O**
Positive Control (with Heparin) E	**O**		**O**
Positive Control (with Heparin) F	**O**		**O**
Positive Control (without Heparin) G	**O**		**O**
Positive Control (without Heparin) H	**O**		**O**

D. Proceed with step 5 from the procedure above.

E. Results: heparin-associated antibodies will be partially or totally inhibited in the presence of excess heparin.

F. If the repeat result (without heparin addition) at the time of heparin inhibition testing is less than 0.4, the flowchart shown in Appendix C should be followed.

G. **NOTE: Appendix C – when performing the confirmation procedure, if the repeat OD (without heparin) is less than 0.4, follow the steps outlined in Appendix C (at the end of the procedure).**

X. CALCULATIONS AND INTERPRETATIONS:

Patient results with a mean OD value of less than 0.400 should be reported as negative. Test results showing mean OD values equal to or greater than 0.400 are regarded as presumptive positive. All presumptive positive samples need re-testing immediately (see confirmation procedure above). This confirmation testing should NOT be delayed until the next day, unless a Pathologist approves a delay in resulting. The term “presumptive positive” should not be used for reporting purposes.

Initial OD	Confirmation Testing % Inhibition by Heparin	Report As
<0.400	Not Applicable	Negative
≥0.400	Greater than 50%	Positive
≥0.400	Less than 50%	Equivocal

XI. INTERPRETATION OF CONFIRMATORY PROCEDURE:

Inhibition of a positive reaction by 50% or more in the presence of excess heparin is considered confirmatory for Heparin-dependent antibody characteristic of Type II HIT. The formula for determining % inhibition follows:

$$\left[(1) - \left(\frac{\text{Patient sample with Heparin}}{\text{Negative Control}} \right) \right] x\ 100 = \%\ \text{Inhibition}$$

Patient sample without Heparin – Negative Control

Example: Patient serum gives an O.D. value of 1.000 in the standard assay with a negative control value of 0.200. With excess heparin, the patient serum gives O.D. value of 0.400. Percent inhibition is:

$$\left[\left(1 - \frac{0.400 - 0.200}{1.000 - 0.200}\right)\right] x\ 100 = 75\%\ \text{Inhibition}$$

The positive control is treated in the same manner as the patient sample. The positive control must be inhibited by >50% after incubation with heparin. Patient results cannot be released without the appropriate control result.

Inhibition of a patient positive reaction by less than 50% is regarded as an equivocal result. This type of reaction is given by a small percentage of antibodies in patients suspected of having Type II HIT. The significance of this type of reaction is not yet established. It has not yet been determined whether it is safe to re-administer heparin to patients whose serum gives an equivocal reaction.[11]
An OD ≥ 2.0 is regarded as a critical value. For patients with critical values, refer to the Calling of STAT, Critical, Corrections and Other Results procedure (OTR.CS.PY.014).

XII. SEND-OUT TESTING PROTOCOL

A. Samples with the following results should be given to the Send-out laboratory by 2 p.m. on the day of testing (Monday through Friday). Appropriate samples from runs performed on Saturday and Sunday should be given to the Send-out laboratory on the following Monday morning. These samples should be stored in the refrigerator in the designated "Send-out" rack until they can be taken to the Send-out laboratory.
    1. Samples with an OD between 0.4 and 1.99 on initial screen independent of confirmatory test result.
    2. Samples with an OD ≥2.0 on initial screen and the confirmatory test result is equivocal.
B. Send-out testing requirements:
    1. A minimum of 1.0 mL of serum is required (1.5 mL is preferred). If the volume is insufficient, determine whether any blood draws have occurred in the prior 12 hours. Blood collected in an SST is acceptable for Serotonin Release Assay (SRA) testing. If additional serum is not available, call the nursing unit or ordering physician and request that an SRA test be ordered directly.
    2. Place the serum into a screw-cap tube, label with the patient information and write “SRA” on the label.

XIII. LIMITATIONS:

A. 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, or omission of steps.
B. 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.

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

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

E. The PF4:PVS complexes used in this assay may differ slightly from those created by PF4:Heparin. Therefore, it is possible that some antibodies could react with PVS complexes that do not react with heparin complexes and vice versa.

F. Although a positive reaction obtained using this assay may indicate the presence of heparin-associated antibody, the detection of such antibodies does not confirm the diagnosis of heparin-induced thrombocytopenia (HIT).

G. Some patients may have naturally-occurring antibodies to PF4.

H. Samples from patients exposed to heparin but not on heparin therapy were not used in the evaluation of this product. Therefore, samples from patients other than those on heparin therapy should not be tested.

I. The following substances have no significant effect (<10% difference in OD values between test sample and control) up to the values listed.

Interfering Substances	
Hemoglobin	500 mg/dL
Triglycerides	500 mg/dL
Bilirubin	20 mg/dL

J. In order to determine possible cross-reactivity between the target antigen and antibodies other than heparin-associated antibodies, 68 samples containing a variety of antibodies which included known antibodies to platelet alloantigens, platelet autoantibodies, antibodies to HLA class I and anti-rheumatoid factor were tested in this assay and none were found to cross react with the target antigen immobilized in the microwells.

XIV. SPECIAL NOTES:

For complete details and performance characteristics of this product, refer to the package insert provided with the test kit.

XV. REFERENCES:

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Attachments

Appendix A: Resulting Negative HPF4 Patient in SOFT
Appendix B: HPF4 Template
Appendix C: Platelet Factor 4 IgG Assay
Appendix D: HPF4 Read on the ELx-800 Plate Reader
Appendix E: HPF4 Read on the ELx-800 Plate Reader
Appendix F: Bio-Tek ELX50 Washer Procedure and Maintenance

Approval Signatures

Step Description	Approver	Date
	Peter Millward: Chief, Clinical Pathology	2/26/2020

Step Description	Approver	Date
Policy and Forms Steering Committee Approval (if needed)	Maria Rivait: Clerkship Coord	2/26/2020
	Timothy Kennedy: Pathologist	1/24/2020
	Gabriel Maine: Dir, Human Leukocyte Antigen	1/24/2020
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Applicability

Royal Oak