Emo-test HIT CONFIRM®

REF 101210-RUO EN

For Research Use Only. Not for Use in Diagnostic Procedures.

Please read the instructions carefully and completely before use

INTENDED USE AND PRINCIPLE

HIT Confirm® is a rapid, one-step, functional cytometric test. **HIT Confirm®** enables the quantification of platelet activation level, in a standardized manner, by detecting two surface markers (one platelet marker, CD41, and one activated platelet marker, CD62) after the plasma has been incubated with a low heparin concentration: (0.3 U/mL) and a high one (100 U/mL). The test is performed with human plasma in studies related to heparin-induced thrombocytopenia (HIT). This kit is for Research Use Only and is not to be used for patient diagnosis or treatment.

MATERIALS

Kit content

Each kit contains enough reagents to carry out 20 individual tests. The color code is also found on the labels and the tube caps.

- 50 mL Dilution buffer
- 110 µL Anti CD41-PE
- 110 µL Anti CD62-FITC
- 250 µL Heparin 3 U/mL
- 250 µL Heparin 1 000 U/mL
- 50 µg TRAP

Not provided

- Plasma sample to be tested
- Healthy donor whole blood (< 6 h)
- Centrifuge
- Tubes suitable for flow cytometry
- Micropipettes and suitable tips
- Vortex
- Timer
- Filters with 0.2 µm membrane
- Incubator (from 20 to 37 °C)
- Flow cytometer

CONSERVATION AND STABILITY

The unopened kit should be stored between 2 and 8 °C and away from light. In these conditions, the reagents are stable until the expiration date indicated on the box label. Except for TRAP that must be reconstituted when used for the first time, all reagents are ready to use. After use, the reagents must be quickly returned to the box and stored between 2 and 8 °C and away from light. Unless contaminated, the opened reagents are stable until the expiration date indicated on the box label. Reagents Anti CD41-PE and Anti CD62-FITC are light sensitive.

PRECAUTIONS

- For Research Use Only
- Do not use the kit after the expiration date.
- Do not use the reagents from another kit. The kit with all reagents must be equilibrated to a temperature between 20 and 25 °C before use.
- Follow carefully the sequence of the steps. Appendix 2: Checklist is used to track and record the completion of each step.
- Comply with Good Laboratory Practices.
- Consider biological material as potentially infectious and wear appropriate personal protection equipment.
- Comply with local regulations for waste disposal.
- Maintain and control the cytometer according to the manufacturer's instructions.
- Ensure that the entire procedure is performed by the same competent person.
- For a result to be validated, each step of the procedure must be completed successfully. Otherwise, the test needs to be repeated with a different PRP.
- The manufacturer or the distributor of the test can be contacted in case of need.

LIMITS

- Healthy donors should not have taken antiplatelet medications (e.g. aspirin), vitamin C and/or anti-inflammatory drugs for at least 7 days prior to blood collection. Other factors may influence platelet biology (e.g. exercise, smoking habit, coffee or alcohol intake).
- Venous blood collection from the donor should be done according to Good Blood Collection Practices. Use only citrated tubes.
- Platelets are not provided with the kit. Each user is responsible for finding healthy donors with reactive platelets. To test platelet reactivity, it is recommended to use a positive HIT plasma as a positive control (not supplied).
- When stored between 20 and 25 °C, donor's whole blood should be used within 6 hours after collection and platelets (PRP) within 3 hours after centrifugation.
- To prevent artefactual platelet activation, PRP handling should be limited to a minimum. Do not vortex the PRP suspension. Homogenize gently by hand before each use to resuspend the platelets.
- It is imperative to filter the sample before use if it has undergone at least one freeze- thaw cycle. This step is essential to remove contaminants that may artefactually activate platelets and/or interfere directly with the cytometric analysis.
- If the plasma sample is frozen to at least - 20 ° C for subsequent tests, single use aliquots of sufficient volume to be filtered should be prepared to avoid freeze-thaw cycles.
- It is the responsibility of each user to determine appropriate and optimal parameters on a given cytometer. Indications are provided in Appendix 1: Cytometer Settings.

PROCEDURE

Plasma sample

1. Collect whole blood on 3.2 % sodium citrate (1 volume citrate for 9 volumes whole blood).
2. Centrifuge at 2 000 g, 10 minutes, between 20 and 25 °C, and stop without using the brake.
3. Transfer the fresh plasma (supernatant) to a new tube without disrupting the lower phase.
4. Maintain the temperature of the plasma between 20 and 25 °C during the procedure.

For subsequent use, single-use aliquots of sufficient volume to be filtered prepared from fresh plasma can be stored below -20 °C. These aliquots should be rapidly thawed at 37 °C before use and then stored between 20 and 25 °C.

Donor's platelet rich plasma (PRP)

5. Collect healthy donor's whole blood on 3.2 % sodium citrate (1 volume citrate for 9 volumes whole blood.
6. Let the blood rest for at least 30 minutes between 20 and 25 °C.
7. Homogenize the blood by turning the tube once slowly and completely.
8. Centrifuge at 200 g, 5 minutes, between 20 and 25 °C, and stop without using the brake.
9. Gently transfer a maximum of PRP (supernatant) to a new tube without disturbing the lower phase and keep between 20 and 25 °C during the procedure.

Caution: Check that the suspension is cloudy or that its platelets count is > 300 000 platelets/µL. Limit excessive PRP handling. Do not mix PRP obtained from different blood samples (even from the same donor). Use blood within 6 hours after collection and PRP within 3 hours after centrifugation.

Reaction

10. When using a kit for the first time, reconstitute TRAP with 50 µL Dilution buffer and homogenize with a vortex until complete dissolution. Otherwise go directly to step 11.

Caution: With the exception of the PRP, which must be gently shaken by hand to resuspend the platelets, each reagent must be homogenized using a vortex and spun. Each reagent should be carefully placed in the bottom of the tube while avoiding the formation of bubbles.

The procedure is summarized in Figure 1.

11. For a plasma sample to be tested, prepare an initial mix consisting of 115 µL Dilution buffer; 5 µL Anti CD41-PE; 5 µL Anti CD62-FITC and 50 µL PRP obtained in step 9.
12. Gently homogenize the initial mix by hand.
13. Label 4 tubes: H0.3; H100; NEG et POS.
14. Distribute 35 µL of the initial mix into each of the 4 tubes.
15. Add specifically to each tube:
    a. H0.3: 5 µL Heparin 3 U/mL and 10 µL plasma sample obtained in step 4.
    b. H100: 5 µL Heparin 1 000 U/mL and 10 µL plasma sample obtained in step 4.
    c. NEG: 15 µL Dilution buffer.
    d. POS: 15 µL Dilution buffer and 2 µL TRAP reconstituted in step 10.
16. Gently homogenize each tube by hand and incubate the 4 tubes in the dark for 30 minutes between 20 and 25 °C.
17. Add 450 µL Dilution buffer to each tube, shake gently to homogenize and immediately read on the cytometer in the following order: NEG; POS; H0.3 and H100.

RESULTS

Cytometer settings

18. When using a cytometer for the first time with the test, adjust the parameters according to Appendix 1: Cytometer Settings. Otherwise go directly to step 19.

Data acquisition

19. Load the HIT Confirm acquisition template (step DD of the Appendix 1).
20. Acquire 10 000 events in « platelets » window with a slow flow rate.
    a. NEG: If necessary adjust the FSC threshold and the vertical marker which defines the " platelets " window.
    b. POS; H0.3; H100: Apply the same parameters as with NEG.

Data analysis

21. Analyze the density dot plots log FSC vs log SSC (Figure 2A). Check that the population is displayed and unique in NEG and POS.
22. Analyze PE histograms. Overlay the population profiles of the 4 tubes (Figure 2B) and check that they are similar in fluorescence intensity. Make sure that at least 95 % of the events are contained in the « platelets» window in NEG and POS.
23. Analyze FITC histograms. Overlay the population profiles of NEG and POS and place a vertical marker at the intersection of the two curves, which delimits on the right the "activated platelets" window according to Figure 2C.
24. Overlay the population profiles of NEG and H100 and check that they are similar in fluorescence intensity and quantity.
25. For each tube, record the percentages of events in the " activated platelets " window.

Caution: Make sure that 2.9 % < %NEG < 19.3 % and that %POS > 84.9 %. If these values are not reached, the test must be repeated with a different PRP.

EMOSIS SAS
11, rue de l'Industrie 67400 Illkirch-Graffenstaden, France,
Tel: +33(0)3.66.32.63.26
www.emosis-diagnostics.com, contact@emosis-diagnostics.com

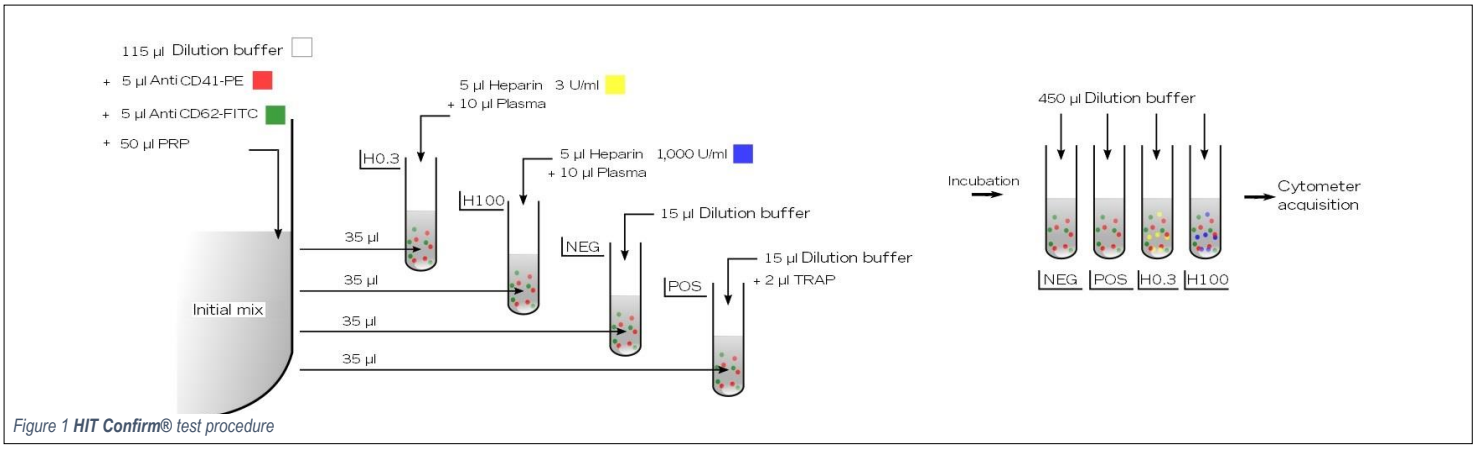
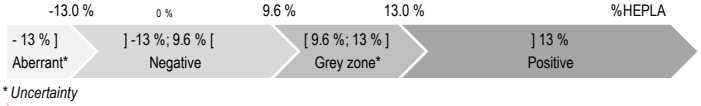


*Figure 1 **HIT Confirm**® test procedure*

Interpretation of results

26. For a given plasma sample, compute the HEparin induced PLAtelet activation Index (%HEPLA) Using the formula: $\%HEPLA = \frac{\%H0.3 - \%H100}{\%POS - \%NEG} \times 100$.
27. Interpret the result according to the % HEPLA value obtained.



** Uncertainty*

Caution: Repeat the test if the result aberrant or in the grey zone..

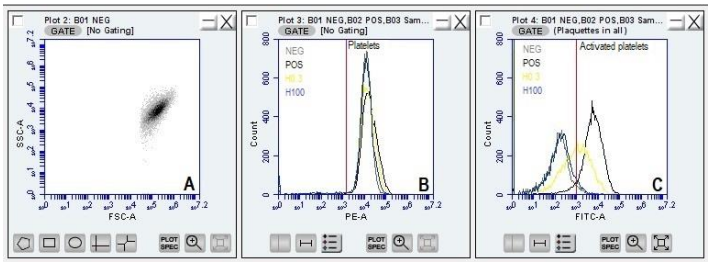


*Figure 2 Acquisition and gating strategy for **HIT Confirm**® test. Typical example of density dot plot log FSC vs log SSC (A) and PE (B) and FITC profiles overlay (C) in the 4 tubes (NEG; POS; H0.3 and H100). Results were obtained on a FACSVia™ cytometer from Becton Dickinson.*

REGISTERED TRADEMARK

ABREVIATIONS

FITC: Fluorescein isothiocyanate
PE: Phycoerythrin
PRP: Platelet rich plasma
SRA: Serotonin release assay
HIT: Heparin induced thrombocytopenia
TRAP: Thrombin receptor agonist peptide

SYMBOLS

Instructions for use
Sufficient for 20 tests
LOT Batch number
Manufacturer
REF Catalog reference
Store between 2 and 8°C
Expiration date

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APPENDIX 1: CYTOMETER SETTINGS

USE

This Appendix should be referred to when using a cytometer for the first time to perform **HIT Confirm®** procedure. It provides indications on how to proceed in setting the parameters. However a competent person in cytometry may use any other equivalent method to achieve the intended results. The parameters to be set are the FSC threshold, voltages and gains and compensations. An acquisition template is defined for subsequent uses on the same cytometer.

PROCEDURE

A. Prepare PRP from donor's whole blood as per steps 5 to 9.
B. If necessary, reconstitute TRAP according to step 10.
C. Prepare a common mix with 190 µL Dilution buffer; 10 µL TRAP and 50 µL PRP.
D. Gently homogenize the common mix by hand.
E. Label 4 tubes: Blank, Double, Mono41 and Mono62.
F. Distribute 50 µL of the common mix into each of the 4 tubes.
G. Add specifically to each tube:
   a. Blank: NA.
   b. Double: 1 µL Anti CD41-PE and 1 µL Anti CD62-FITC
   c. Mono41: 1 µL Anti CD41-PE
   d. Mono62: 1 µL Anti CD62-FITC.
H. Gently homogenize each tube by hand and incubate the 4 tubes in the dark for 30 minutes between 20 and 25 °C.
I. Add 450 µL Dilution buffer to each tube, gently homogenize and read immediately on the cytometer.

CYTOMETER SETTINGS

Light scatter

J. Display a density dot plot log FSC vs log SSC.
K. Acquire 10 000 events in « Double » tube with a slow flow rate while testing different FSC thresholds until you get the display of Figure 3A.
L. Set this value as the FSC threshold.
M. Adjust voltages and gains until you get the desired population centered within the window as shown in Figure 3A.

Fluorescence: Amplification

N. Display a PE histogram and a FITC histogram.
O. Acquire 10 000 events in « Double » tube with a slow flow rate while testing different voltages and gains, on PE and on FITC, until you get populations with intermediate fluorescence intensity as shown in Figures 3B and 3C.
P. Apply the previously defined voltages and gains, and acquire 10 000 events in « Blank » tube with a slow flow rate. Check that the populations have lower fluorescence intensities but are still completely displayed in the window. Otherwise, repeat step O.
Q. Set these values as amplifications parameters (voltages and gains).

Fluorescence: Compensation

R. Apply the previously defined FSC threshold, and voltages and gains for FSC, SSC, PE and FITC. Delete all compensations.
S. Display a PE histogram and a FITC histogram.
T. Acquire 10 000 events, slow flow rate, in tubes: Blank, Mono41 and Mono62.
U. Record median fluorescence intensities (MFI): MFI PE on PE histogram and MFI FITC on FITC histogram.
V. Correct FITC by removing obtained %PE and correct PE by removing obtained %FITC:

$$\%PE = \frac{\text{MFI PE Mono62 - MFI PE Blank}}{\text{MFI FITC Mono62 - MFI FITC Blank}} \times 100$$

$$\%FITC = \frac{\text{MFI FITC Mono41 - MFI FITC Blank}}{\text{MFI PE Mono41 - MFI PE Blank}} \times 100$$

W. Set these values as compensations on PE and FITC.

ACQUISITION TEMPLATE

X. Display a density dot plot log FSC vs log SSC, a PE histogram and a FITC histogram.
Y. Apply the previously defined FSC threshold, voltages and gains for FSC; SSC; PE and FITC. Apply the compensations PE and FITC.
Z. Acquire 10 000 events in « Double » tube with a slow flow rate.
AA. On PE histogram, set a vertical marker at the base of PE^+ population that delimits on the right the "platelets" window according to Figure 4B.
BB. On FITC histogram, activate « platelets » window.
CC. Prepare a template containing 4 readings: NEG; POS; H0.3 et H100.
DD. Save the HIT Confirm template for subsequent uses.

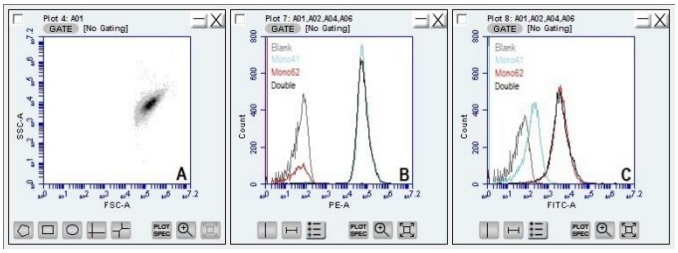

*Figure 3 Cytometer settings strategy for **HIT Confirm®** test. Typical example of density dot plot log FSC vs log SSC (A) and PE (B) and FITC profiles overlay (C) in the 4 tubes (Blank; Mono41; Mono62; Double). Results were obtained on a FACSVia ™ cytometer from Becton Dickinson.*

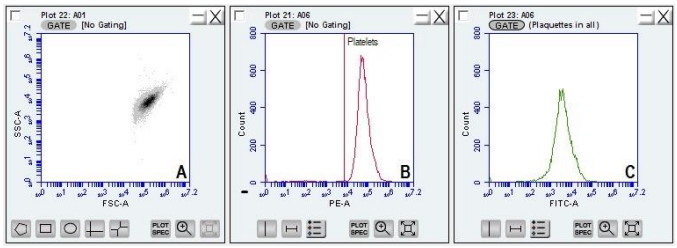

*Figure 4 Acquisition and gating strategy for **HIT Confirm®** test. Typical example of density dot plot log FSC vs log SSC (A) and PE (B) and FITC profiles overlay (C). Results were obtained on a FACSVia ™ cytometer from Becton Dickinson.*

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APPENDIX 2: CHECKLIST

Caution: To validate a result in accordance with this procedure, each step must be successfully completed. Otherwise, the test will need to be repeated with a different PRP. The entire procedure is carried out between 20-25 °C.

KIT
Batch: _____ Expiration date: _____
Number of previous uses: _____
☐ Store between 2 and 8 °C

CYTOMETER AND SETTINGS *(See Appendix 1 when using a cytometer for the first time)*
Model: _____ ☐ Perform daily QC
☐ Record the values of the settings:

	FSC	SSC	PE	FITC
☐ Threshold		/	/	/
☐ Voltages / Gains				
☐ Compensation	/	/	___ % FITC	___ % PE

☐ Acquisition template name: _____

PLASMA SAMPLE
Identification: _____

• If fresh plasma
☐ Collect whole blood on 3.2 % sodium citrate, Date/Time: _____
☐ Centrifuge 2 000 g, 10 min, between 20 and 25 °C, stop without using the brake.
☐ Transfer supernatant to a new tube without disrupting the lower phase.
☐ Use fresh after vortex.
☐ Prepare single-use aliquots and freeze for a subsequent use.

• If frozen plasma
Freezing date: _____ ☐ -20 °C ☐ -80 °C
☐ Thaw rapidly at 37 °C.
☐ Filter the plasma on a 0.2 µm membrane.

DONOR'S PLATELETS (PRP)
Identification healthy donor: _____
• *Whole blood*
☐ Collect whole blood on 3.2 % sodium citrate, Date/Time: _____
☐ Let the blood rest at least 30 minutes: From _____ to _____
☐ Use within 6 hours after collection

• *PRP*
☐ Centrifuge 200 g, 5 min, between 20 and 25 °C, stop without using the brake, Time: _____
☐ Transfer supernatant to a new tube without disrupting the lower phase
☐ PRP is cloudy,
Platelet count _____, Approximate volume: _____
☐ Use within 3 hours after centrifugation

REACTION
• *TRAP Reconstitution (first use of a kit)*
☐ Add 50 µL Dilution buffer
☐ Vortex until complete powder dissolution

• *Initial mix (IM)*
☐ Label a tube with: IM
☐ Gently shake PRP by hand and vortex and spin all kit reagents
☐ Add 115 µL Dilution buffer, ☐ 5 µL Anti CD41-PE, ☐ 5 µL Anti CD62-FITC and ☐ 50 µL PRP
☐ Gently shake by hand

• *H0.3; H100; NEG; POS*
☐ Label 4 tubes: H0.3; H100; NEG and POS
☐ Gently shake Initial Mix by hand and vortex plasma sample
☐ Add:

	H0.3	H100	NEG	POS
Initial mix	35 µL ☐	35 µL ☐	35 µL ☐	35 µL ☐
Heparin 3 U/mL 🟨	5 µL ☐	/	/	/
Heparin 1 000 U/mL 🟦	/	5 µL ☐	/	/
Plasma sample	10 µL ☐	10 µL ☐	/	/
Dilution buffer	/	/	15 µL ☐	15 µL ☐
TRAP	/	/	/	2 µL ☐

☐ Gently shake all tubes by hand
☐ Incubate the 4 tubes in the dark for 30 minutes: from _____ to _____
☐ Add 450 µL Dilution buffer to each tube and gently shake
☐ Read on the cytometer, immediately.

ACQUISITION
☐ Load HIT Confirm template
☐ Follow the reading sequence NEG; POS; H0.3 and H100.
☐ NEG: Acquire 10 000 events in « platelets » window with a slow flow rate.
☐ If needed adjust the FSC threshold ☐, and the « platelets » window ☐
☐ POS ☐ H0.3 ☐ H100; Apply the same parameters as with NEG.

ANALYSIS
• Density Dot plot log FSC vs log SSC.
Check the population in NEG and POS: ☐ Displayed ☐ Unique

• PE Histograms
Overlay the population profiles of the 4 tubes and check that:
☐ Similar in fluorescence intensity
☐ > 95 % in « platelets » ☐ NEG ☐ POS

• FITC Histograms
☐ Overlay NEG and POS
☐ Set a vertical marker at the intersection of the two curves.
☐ Overlay NEG and H100, ☐ Similar in fluorescence intensity
☐ Record the percentages of events in the « activated platelets ».

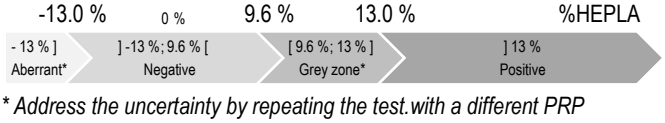
%NEG = _____% %POS = _____%
☐ Confirm: 2.9 % < %NEG < 19.3 % and %POS > 84.9 %

%H0.3 = _____% %H100 = _____%

INTERPRETATION

$$\%HEPLA = \frac{\%H0.3 - \%H100}{\%POS - \%NEG} \times 100 = \frac{\qquad}{\qquad} = \_\_\_\_\_\%$$

☐ Interpret the result according to %HEPLA value



** Address the uncertainty by repeating the test.with a different PRP*

☐ Negative ☐ Positive ☐ To be repeated

Visa: _____Date: _____

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