Emo-test HIT Confirm®

REF 101210-RUO EN

In vitro test kit to quantify the platelet activation level induced by heparin in plasma samples

DESCRIPTION

Warning! Read the entire instruction manual before use

The operating instructions are also available in electronic form at: www.emosis-diagnostics.com

ABBREVIATIONS

FITC	Fluorescein isothiocyanate (fluorochrome)
PE	Phycoerythrin (fluorochrome)
PRP	Platelet Rich Plasma
PPP	Platelet Poor Plasma
SRA	Serotonin Release Assay
TIH	Heparin-Induced Thrombocytopenia
TRAP	Thrombin Receptor Agonist Peptide
%R	Percentage of platelet activation

SYMBOLES

- See instructions for use
- Sufficient for testing 20 PRPs individually
- LOT Batch number
- REF Catalog number
- Storage between 2 and 8°C
- Expiry date
- Manufacturer

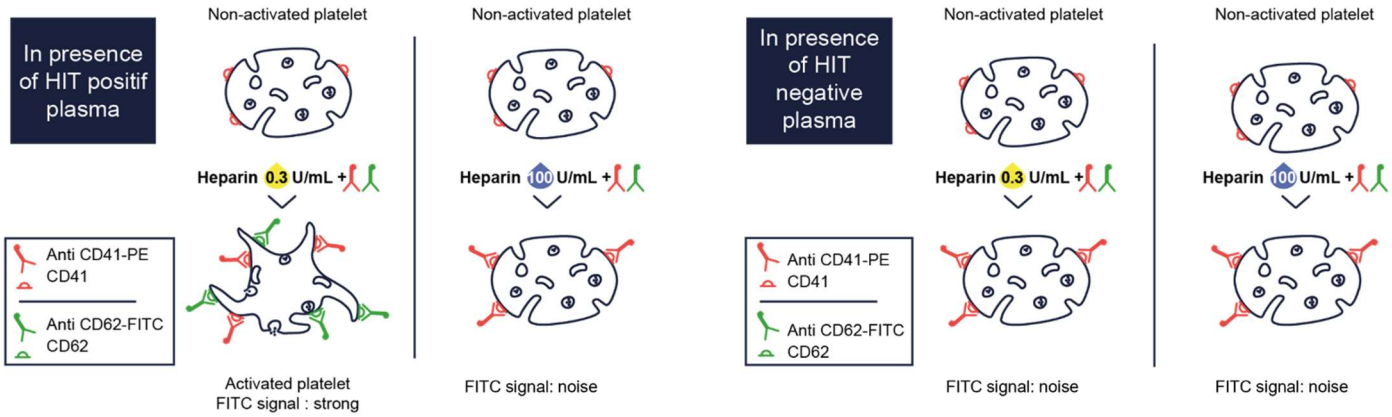

Figure 1 : Principle of test

INTENDED USE

HIT Confirm® is a flow cytometry-based rapid functional test which enables the quantification of immune-mediated platelet activation induced by heparin.

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.

PRINCIPLE

HIT Confirm® quantifies in a normalized way the differential activation of platelets induced by plasma of plasma samples exposed to low and high concentrations of heparin. This quantification is based upon the measure of platelet activation change when a therapeutic level of heparin (0.3 U/mL) is used as compared to a supratherapeutic level of heparin (100 U/mL). Platelet activation is detected by using monoclonal antibodies (MoAbs) against specific surface markers (platelet marker CD41 and activated platelet marker CD62). These MoAbs are conjugated with fluorophores (anti CD41-PE, anti CD62-FITC) that reemit when illuminated by laser beam.

The result of HIT Confirm is expressed by the ratio of the difference of percentages of platelet activation induced by each of heparin concentration, divided by the maximum of activation triggered by a potent platelet agonist. This ratio is called HEPLA.

EMOSIS SAS
11 rue de l'industrie, 67400 Illkirch-Graffenstaden, France
Tél : +33 (0)3 66 32 63 26
www.emosis-diagnostics.com, contact@emosis-diagnostics.com

Version 3 du 02/06/2022

GENERAL PRECAUTIONS

- Do not use the kit after the expiration date
- Do not use or mix reagents from another kit or batch number
- The kit must be equilibrated to a temperature between 20 and 25°C before use for 60 minutes
- Comply with Good Laboratory Practices
- Carefully follow the sequence of the steps
- Comply with local regulations for waste disposal
- Ensure that the entire procedure is performed by the same trained person
- All reagents with an unusual appearance or contamination must be eliminated
- TRAP must be reconstituted when using for the first time, all reagents are ready to use

LIMITS

Possible false negative results were reported with a functional assay (HIPA) in patients treated with Ticagrelor[1].

[1]Julia J. M. Eekels, Christoph Pachler, Nora Krause, Tina Muhr, Gert Waltl, and Andreas Greinacher. To the editor: Ticagrelor causes false-negative functional tests for heparin-induced thrombocytopenia. Blood 12 MARCH 2020 | VOLUME 135, NUMBER 11 8

CONSERVATION AND STABILITY

- The kit must be stored between 2 and 8°C
- The kit must be kept away from light, (anti CD41-PE and anti CD62-FITC are light-sensitive)
- After use, the reagents must be quickly returned to the box to be stored between 2 and 8°C in the dark
- To avoid any risk of evaporation of the reagents, close them after use

In these conditions, the reagents are stable until the expiration date indicated on the box label

EQUIPMENTS AND REAGENTS

Kit content

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

Equipment not provided

- Flow cytometer equipped with blue laser
- Tubes suitable for flow cytometry
- Centrifuge
- Incubator
- Timer
- Vortex
- Micropipettes and suitable tips

Reagents not provided

- Plasma sample to be tested
- Healthy donor's whole blood
- HIT positive control

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SAMPLE COLLECTION *(See details in PROCEDURE HIT Confirm® below)*

Warning
Veinous blood drawing from blood donors must comply with good practice and blood samples must be collected in citrated tubes only.

Precautions for blood donors
Before collecting blood, verify that donors neither took antiplatelet drugs such as aspirin vitamin C and/or anti-inflammatory drugs at least for the previous 7 days.

QUALIFICATION OF PRP

Platelets must be qualified either by using platelets from selected consenting donors whose platelets are known to be reactive when used to performed HIT functional assays, or by validating platelets from unselected donors against known HIT positive sample or HIT mimicking antibody (e.g., 5B9).

SAMPLE PREPARATION

« Plasma » sample

Precaution
Consider biological material as potentially infectious and wear appropriate personal protection equipment (gloves, mask, glasses, lab coat, …).

Donors' (PRP)
Warning! At least 2 different PRPs are to be used

Warning

- Donors' blood must be used within 6 hours after blood draw when kept at room temperature (between 20 and 25°C)
- Donors' platelets must be used within 3 hours after centrifugation, and must be stored at room temperature (between 20 and 25°C)
- Restrict as much as possible the manipulation of PRP to avoid artefactual action of platelets

Frozen samples

Warning

If samples are stored frozen for further testing, there are two options:
1.Filter them with 0.2 µm microfilters (not provided) and split in single use aliquots of enough volume to be tested (e.g., 300µL for 10 aliquots of 22µL).
2. Filter enough to perform a test once the sample is defrosted. This way avoids quality deterioration of samples due to freeze-thaw cycles.

Possibility of batch testing
The method allows the testing of a batch of maximum 5 plasma samples per PRP.

CYTOMETER

Precautions

- It is up to each laboratory to define optimal parameters for a given flow cytometer (see *APPENDIX: CYTOMETER* below).
- Maintenance and control of flow cytometer must be compliant with manufacturer's instruction.

INCIDENTS

Any serious incidents associated with the kit must be notified to the manufacturer and to the relevant health authority of manufacturer's country (Agence Nationale de la Sécurité des Médicaments et des Produits de Santé : ANSM) EMOSIS SAS can be contacted directly through : contact@emosis-diagnostics.com.

ANALYTICAL PERFORMANCES

Repeatability

HEPLA positive samples (+)	CV H0.3	CV H100	CV HEPLA (+)
CV mean	9 %	22 %	8 %
CV min	6 %	13 %	5 %
CV max	13 %	28 % (1)	11 %

HEPLA negative samples (-)	CV H0.3	CV H100	CV HEPLA (-)
CV mean	14 %	20 %	25 %
CV min	6 %	4 %	16 %
CV max	25 %	35 % (1)	44 %

Reproducibility

HEPLA positive samples (+)	CV H0.3	CV H100	CV HEPLA (+)
CV mean	12 %	39 %	15 %
CV min	11 %	31 %	11 %
CV max	13 %	46 % (1)	19 %

HEPLA negative samples (-)	CV H0.3	CV H100	CV HEPLA (-)
CV mean	18 %	25 %	33 %
CV min	7 %	11 %	16 %
CV max	28 %	35 % (1)	73 %

Minimum and maximum values obtained for %R H100 in repeatability and reproducibility studies:

Samples	%R H100 min	%R H100 max
HEPLA positive (+)	8 %	17 %
HEPLA negative (-)	7 %	18 %

PROCEDURE HIT Confirm®

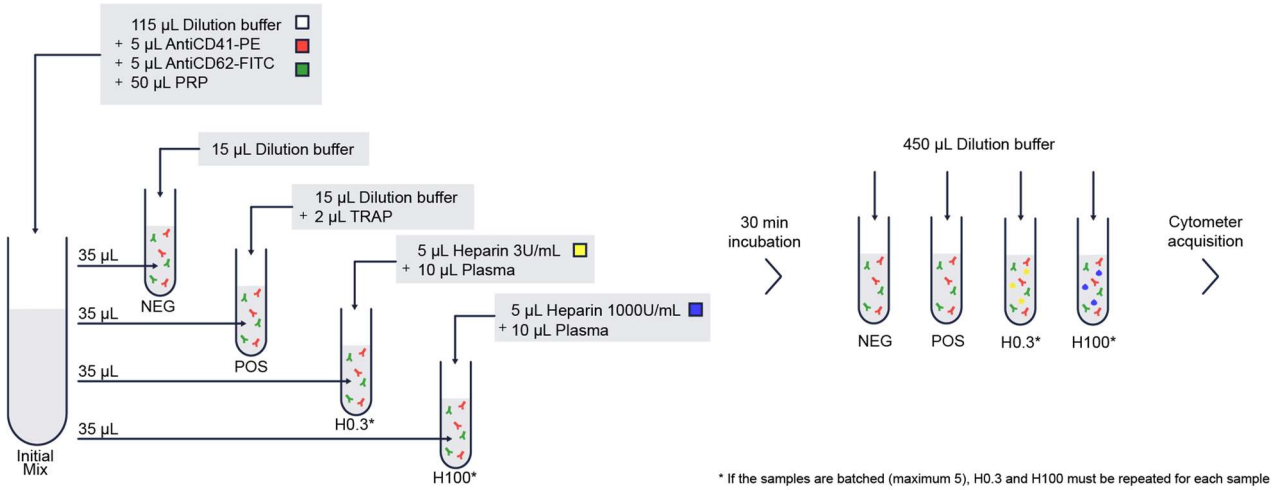

Figure 2 : Test procedure

PREREQUISITE

To be validated, each step must be successfully performed otherwise, the assay must be repeated.
Procedure is performed between 20 et 25°C with the kit temperature being stabilized accordingly before proceeding with the assay.

PLASMA SAMPLE

If using fresh plasma:

☐ Collect whole blood on sodium citrate 3,2%

☐ Centrifuge: 2000 g, 10 minutes, between 20 and 25°C, without brakes

☐ Collect plasma in a tube without disturbing inferior phase

☐ Prepare single use aliquots for storing for future sample testing, if necessary

If using frozen plasma:

☐ Unfreeze sample at 37°C

☐ Filter plasma with 0,2 µm porosity microfilter, if necessary

QUALIFIED DONOR'S PLATELETS (PRP)

Whole blood

☐ Collect whole blood with a sodium citrate 3,2% tube

☐ Let the whole blood sample to stand for at least 30 minutes

PRP preparation

☐ Centrifuge: 200 g, 5 minutes, between 20 and 25°C, without brakes

☐ Collect all PRP in a tube without disturbing inferior phase

☐ Perform platelet count

☐ Use within 3 hours after centrifugation

PPP preparation

☐ Centrifuge citrated tube from previous step: 2000 g, 10 minutes, between 20 and 25°C, without brakes

☐ Collect PPP in a tube without disturbing inferior phase

☐ Dilute PRP with PPP to obtain about 300 000 platelets/µL

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REACTION

Reconstitute TRAP (1st kit use only)
☐ Add 50 µL of dilution buffer
☐ Vortex solution to dissolve powder totally

Initial Mix (IM)
☐ Label a tube
☐ Vortex kit reagents and gently shake PRP by hand
☐ Prepare Initial Mix as described below

		Preparation of Initial Mix (IM)			
		Dilution buffer (µL)	**Anti-CD41 MoAb (µL)**	**Anti-CD62 MoAb (µL)**	**PRP (µL)**
Color code		☐	🟧	🟩	N/A
Number of sample(s)	1	115	5	5	50
	2	161	7	7	70
	3	207	9	9	90
	4	253	11	11	110
	5	299	13	13	130

☐ Gently shake by hand

Reactional tubes
☐ Prepare required tubes
☐ Vortex plasma sample
☐ Gently shake initial mix by hand

☐ Add:

	NEG	POS	H0,3*	H100*
Initial Mix	35 µL			
Heparin 3 U/mL 🟨	/	/	5 µL	/
Heparin 1000U/mL 🟦	/		/	5 µL
Plasma sample	/	/	10 µL	
Dilution Buffer	15 µL		/	/
TRAP	/	2 µL	/	/

**To repeat according to the number of samples*
☐ Close the tubes
☐ Gently shake each tube by hand
☐ Incubate 30 minutes, in the dark
☐ Add 450 µL of dilution buffer in each tube
☐ Gently shake all tubes by hand

SIGNAL AQUISITION

☐ Load HIT Confirm template
☐ Verify cytometer parameters: 10 000 events in « platelets » at slow flow, threshold
☐ Read tubes in the following order: NEG; POS; (H0.3 and H100) x as many times as there are plasma samples
☐ If needed adjust threshold of FSC and « platelets » window and read again

ANALYSIS

☐ Verify dot plot log FSC vs log SSC to check for unusual platelet population pattern

☐ Verify histogram PE to check for consistency of fluorescence intensity in all tubes

☐ Superimpose NEG and POS FITC histograms corresponding to platelet population only

☐ Position a vertical line at the intersection between NEG and POS

☐ Record % of events in « activated platelets » window for all tubes

%NEG = _____ %
%POS = _____ %

☐ Respected limits: 2.9% < %NEG < 19.3% and % POS > 84.9%

INTERPRETATION *See Figure 3*

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

If %HEPLA is above 13% test result is considered positive.
If %HEPLA is below 9.6%, %H100 must be verified.

If %H100 is high, above 23%, result is considered indeterminate, and the test must be repeated with another PRP.

If %H100 is below 23%, result is considered negative.

If %HEPLA is in the gray zone (between 9,6% and 13%), result is considered indeterminate, and the test must be repeated with another PRP.
If %HEPLA remains indeterminate after several repeats, the test is considered as non-conclusive.
In case of discordant results among repeats, the « believe the positive » rule applies.

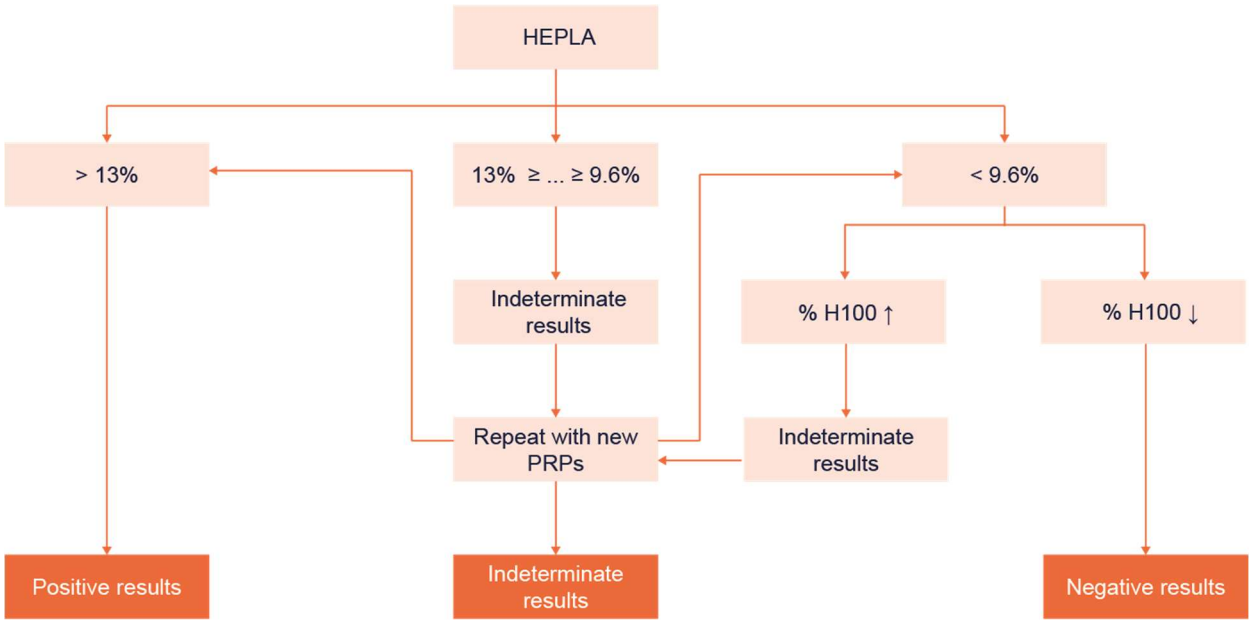

Figure 3 : Test interpretation

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APPENDIX: CYTOMETER

PURPOSE

This appendix must be read before the first use of HIT Confirm on a given flow cytometer. It provides suggestions for the appropriate settings for the flow cytometer. A qualified operator in flow cytometry may set alternative tuning to achieve the same outcome. A signal acquisition template will be defined based on the selected settings, to be reused for further testing.

PROCEDURE

- Obtain donors' platelets (PRP) according to sample preparation described above.
- Reconstitute TRAP solution as follow: 50µL of dilution buffer and vortex
- Prepare a common mix made of 190 µL of dilution buffer, 10 µL of TRAP and 50 µL of PRP.
- Homogenize the common mix gently by hand
- Label 4 tubes : Blank, Double, Mono41 et Mono62
- Distribute 50 µL of the common mix in each of the 4 tubes
- Add, specifically to each tube:
  Blank: N/A
  Double: 1 µL Anti CD41-PE and 1 µL Anti CD62-FITC
  Mono41: 1 µL Anti CD41-PE
  Mono62: 1 µL Anti CD62-FITC
- Homogenize gently by hand and incubate the 4 tubes during 30 minutes between 20 and 25°C, in the dark.
- Add 450 µL of dilution buffer in each of the 4 tubes and read immediately with the cytometer display.

CYTOMETER SETTING

Size / Structure

- Display density dot plot log FSC vs log SSC
- Read 10 000 events at slow rate in the tube Double, while testing different FSC threshold until getting display such as in Figure 4 picture A
- Retain the value as FSC threshold
- Adjust voltages and gains until the platelet population is positioned on the center of the window of interest Figure 4 picture A

Fluorescence: Amplification

- Plot a PE histogram and a FITC histogram
- Read 10 000 events at slow flow in the tube Double, while testing different voltages and gains, on PE and on FITC, until getting populations displaying medium range intensity, as in examples of Figures 5 pictures B and C.
- Read 10 000 events at slow flow rate in the tube Blank, applying priorly defined voltage and gain.
- Verify that the fluorescence intensity of the population is lower than that of the positive population, but is fully contained within the gated window
- Fix voltage and gain values.

Fluorescence: Compensation

- Apply threshold, voltages and gains defined for FSC; SSC; PE et FITC. Delete compensations.

Depending on the flow cytometer brand and model, some of the following steps can be done automatically. If not, here is a proposed method of compensation:

- Display PE histogram and FITC histogram
- Read 10 000 events at slow flow rate in tube Blank: Mono41 et Mono62
- Record the median fluorescence intensity (MFI): MFI PE on PE histogram and MFI FITC on FITC histogram
- Fine tune FITC by eliminating resulting %PE and fine tune PE by eliminating resulting %FITC:

$$\%PE = \frac{\text{MIF PE Mono62 - MIF PE Blanc}}{\text{MIF FITC Mono62 - MIF FITC Blanc}} \times 100$$

$$\%FITC = \frac{\text{MIF FITC Mono41 - MIF FITC Blanc}}{\text{MIF PE Mono41 - MIF PE Blanc}} \times 100$$

- Fix these values as compensation values

ACQUISITION TEMPLATE

- Display density dot plot of log FSC vs log SSC, PE histogram and FITC histogram
- Apply FSC threshold, voltages and gains defined priorly for FSC; SSC; PE et FITC, and compensations for PE and FITC
- Read 10 000 events at slow flow rate in tube Double
- On PE histogram position a vertical line at the left extreme end of PE+ histogram to define « platelets » on the right side of the demarcation line as in figure 5 picture B
- On FITC, activate « platelets » window
- Prepare a template for reading: NEG; POS; H0,3; H100

Save HIT Confirm template for further testing

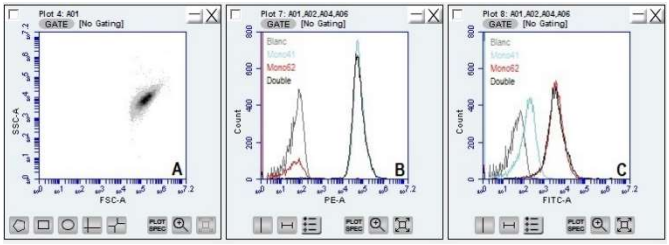

*Figure 4: Strategy of flow cytometer parameter setting for **HIT Confirm®**. Illustrating example of density plot of log FSC vs log SSC (A) and superimposition of PE (B) and FITC (C) from 4 tubes (Blank; Mono41; Mono62; Double). Results obtained FACSVia™ from Becton Dickinson*

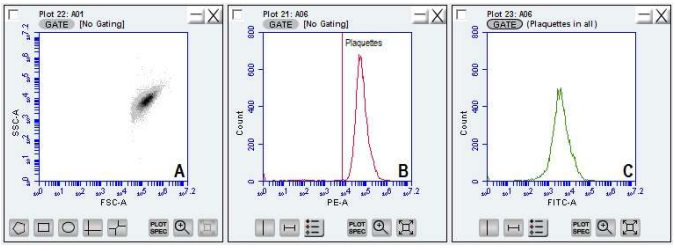

Figure 5: Acquisition and gating strategy for HIT Confirm®. Example of density dot plot of log FSC vs log SSC (A) and superimposition of PE (B) and FITC (C) from 4 tubes (Blank; Mono41; Mono62; Double). Results obtained with FACSVia™ from Becton Dickinson

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