HIT*Alert*™ Kit

REF IQP-396-USA   30 tests   package insert

RUO ***Research Use Only.***

Not for use in diagnostic procedures.

PACKAGE INSERT

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HIT*Alert*™ Kit

The HIT*Alert*™ Kit is used for the detection of heparin complex specific antibodies.

Kit content

Reagent A	Assay buffer	5 ml
Reagent B	Heparin	150 µl
Reagent C	Platelet Activator (Ca-Ionophore)	1 vial
Reagent D	Staining buffer	20 ml
Reagent E	Platelet marker (Monoclonal antibody)	200 µl
Reagent F	Platelet Activation marker (Recombinant Protein)	200 µl
Reagent G	Heparin 1000 U/ml	150 µl
2.2 ml PP vials used for the sample incubation		30

Each kit contains sufficient reagents to test 6 test samples (30 tests).

Laboratory material required but not included

- Flow cytometer
- citrate blood collection tube, for instance Greiner Vacuette 454382
- Tubes fitting the flow cytometer
- 70% or 96% ethanol
- Shaker (for instance ELISA plate shaker or platelet agitator)
- Laboratory centrifuge
- Adjustable micropipettes and tips

Storage

Upon receipt, store kit at 2 to 8 °C. Avoid direct sunlight. Reagents stored according to stated storage instructions are stable until the expiration date indicated on the label. For repeatedly testing store the reagents immediately after usage at 2 to 8 °C and the dissolved reagent C at -20 °C.

Warning and precautions

For Research Use Only. Reagents containing sodium azide may react with lead or copper plumbing to form explosive metal azides. On disposal, flush with large amounts of water to prevent azide build-up. All reagents should be handled in accordance with good laboratory practices using appropriate precautions. In addition, handle all test samples with appropriate precautions. Do not pipette by mouth and wear gloves during the procedure.
Substitution of components other than those provided in this kit may lead to inconsistent or erroneous result.
The test must be performed by well-trained and authorized laboratory technicians. Please contact the manufacturer when the original kit is damaged.

Instrument Requirements

- Make sure that the flow cytometer is calibrated correctly according to manufacturer's instruction.
- It is advised to perform instrument calibration and maintenance on regular basis.
- The flow cytometer should be operated by a technician skilled in the art. Evaluation of the results should be done by someone skilled in the interpretation of flow cytometric data.

Specimen Collection and Preparation

Platelet rich plasma (PRP)

Not all platelet donors have platelets suitable to make PRP to use for functional assays. PRP from several different donors should be tested with a sample known positive for HIT. The donor most suitable will give the highest activation in sample IV (sample with physiological concentration of heparin). It also important to screen the same platelet donors with a sample from individuals known to be negative for HIT.

It is important that the platelet donor did not use platelet inhibitors, like aspirin, or anti-inflammatory drugs, like Ibuprofen, Advil, etc. during the last 3 to 4 days prior to the blood draw. These agents can cause failure of the assay, although reagent C may still work well.

Preparation of platelet rich plasma (PRP)

- Collect venous blood of a 0 blood type donor into a Citrate Solution Evacuated Tube (for instance: 454382, Greiner Vacuette), using aseptic venapuncture.
- Mix the blood with the citrate once by gently inverting the tube. *Prevent unnecessary agitation*.
- The blood sample should be stored at room temperature (20 to 25 °C) and processed directly after drawing.
- Spin the blood 5 minutes at 100g with low acceleration and brake off.
- Remove the cap and collect the upper yellow fluid, this is the PRP, into an empty tube. Stay well above the red and white cell pellet! WBC and RBC are inconvenient in the test.
- Use the PRP within 2 hours.

Test Procedure HIT*Alert*™ Kit

Do *not* use pre-warmed or heated (heat inactivated) test samples.

1. Dissolve reagent C in 200 µl 70% or 96% ethanol. This is the reagent C stock solution.
2. Mix the reagent C stock solution well by vortexing or end over end mixing.
3. Reagent C needs 15 to 30 minutes to dissolve completely in ethanol. The dissolved material might show a little precipitation.
4. This reagent C stock solution can be used directly for the assay. Mark the vial properly and store the reagent C stock solution after use at -20 °C, so it can be used for the next assays from the same kit too.

Sample incubation

5. It is important that the steps are executed in the right order and with care. Abrupt agitation will decrease the reliability of the assay.
6. Perform the first incubation step (table 1) in the 2.2 ml vials included in the kit.
7. Perform the assay by adding together the reagents from left to right on the bottom of a 2 ml vial (*Make sure you use a new tip with every pipetting step*):

Assay Samples	Reagent A	PRP	Test Sample	Reagent B	Reagent G	Reagent C
I	35 µl	10 µl	---	5 µl	---	---
II	35 µl	10 µl	---	---	---	5 µl
III	30 µl	10 µl	10 µl	---	---	---
IV	25 µl	10 µl	10 µl	5 µl	---	---
V	25 µl	10 µl	10 µl	---	5 µl	---

Table 1. Components to add together for sample incubation.

I: PRP with heparin
II: PRP with calcium ionophore
III: PRP with test sample
IV: PRP with test sample and heparin
V: PRP with test sample and 100 U/ml heparin

8. Mix the suspension carefully by pipetting up and down. *Avoid generating air bubbles.*
9. Incubate the tubes at room temperature (20 to 25 °C) on a horizontal orbital shaker for one hour. The speed of the shaker must be fast enough to get a slight movement of the samples. *Avoid the generation of air bubbles.*

Staining of the samples

10. Prepare 5 tubes (suitable for flow cytometry) by labeling them with I, II, III, IV and V.
11. Make in a new tube a mixture of 210 µl reagent D, 30 µl reagent E and 30 µl reagent F and mix well.
12. Add 45 µl of this mixture to each of the tubes from step 10. Store the tubes in the dark until part of the incubated assay sample can be added.
13. After the one hour incubation (step 9) add 5 µl of the assay sample mixture to the corresponding tube with staining solution. Mix the samples by carefully pipetting up and down. *Avoid generation of air bubbles.* Incubate the mixture 15 minutes in the dark at room temperature (20 to 25 °C).
14. Add 400 µl reagent D to the tube.
15. The cells are now ready for acquisition and evaluation by flow cytometry. Please run acquisition as soon as possible and no later than 30 minutes after addition of reagent D.

Data collection

Adjustment of flow cytometer

For adjustment of the flow cytometer settings three tubes are used (table 2):

- Label three tubes (suitable for the cytometer) with S1, S2 and S3.
- Add the different components to the tubes following table 2. Make sure to add 5 µl assay sample II in each tube.
- Mix the tubes carefully by pipetting up and down avoid generating of air bubbles. Incubate 15 minutes in the dark at RT.
- Add 400 µl reagent D to each tube.
- Cells are now ready to use for set up of the flow cytometer.

Tube	Samples (5 µl)	Reagent D	Reagent E	Reagent F
S1	II	45 µl	---	---
S2	II	40 µl	5 µl	---
S3	II	40 µl	---	5 µl

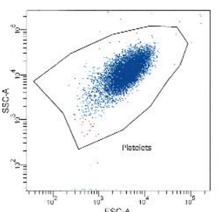
Table 2. Components to add together for adjustment of the settings of the flow cytometer. Platelets are used from assay sample II (step 4).

Analysis

1. Make three dot plots, a Forward scatter (FSC) vs. Sideward Scatter (SSC) dot plot with logarithmic scale to select the platelets, a R-PE vs. SSC dot plot to select the platelet marker positive events and a FITC vs. R-PE dot plot to determine the activation of the platelets.

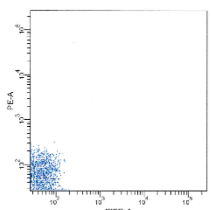
2. Adjust the voltage settings for the FSC-SSC by use of tube S1.

   Select all platelets by using a region and exclude debris and background noise by setting the appropriate FSC threshold (see Cytogram 1). Do *not* make the gate too tight on the lower left hand side. After activation of the platelets part of the platelets will get smaller (microvesicles). This gate can be checked by using backgating of the activation marker and platelets marker positive cells from step 5. Activate the region for the next step in the evaluation.



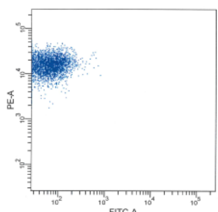
Cytogram 1. SSC (vertical) / FSC (horizontal) dot plot and region to select the platelets.

Sample S1 is also used to adjust the FL-1 and FL-2 photomultiplier tube (PMT) voltages. Make a FL-1/Fl-2 dot plot and set the FL-1/FL-2 baseline signals in the lower left corner in an FL-1 vs. FL-2 dot plot (see Cytogram 2).

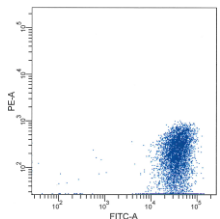


Cytogram 2. Correct adjustment of the PMT voltages for FL-2 (vertical) and FL-1 (horizontal) of the unstained sample.

3. Sample S2 and S3 are used for adjusting the compensation. These compensation settings between FITC (FL-1) and R-PE (FL-2) fluorescence signals should be optimized to separate the stimulated (FL-1 positive) from the unstimulated (FL-1 negative) platelets correctly.
   a. use sample S2 to adjust the compensation of R-PE (FL-2) from the FITC (FL-1) channel (see Cytogram 3).
   b. use sample S3 to adjust the compensation of FITC (FL-1) from the R-PE (FL-2) channel (see Cytogram 4).



Cytogram 3. Compensation of R-PE (FL-2, vertical) signal from the FL-1 (horizontal) channel.

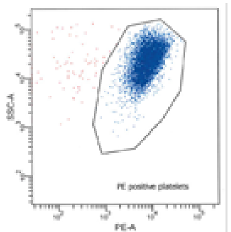


Cytogram 4. Compensation of FITC (FL-1, horizontal) signal from the FL-2 (vertical) channel.

4. Finally the assay samples can be analyzed one by one after selecting the platelet marker (FL-2) positive events in a SSC / FL-2 dot plot.

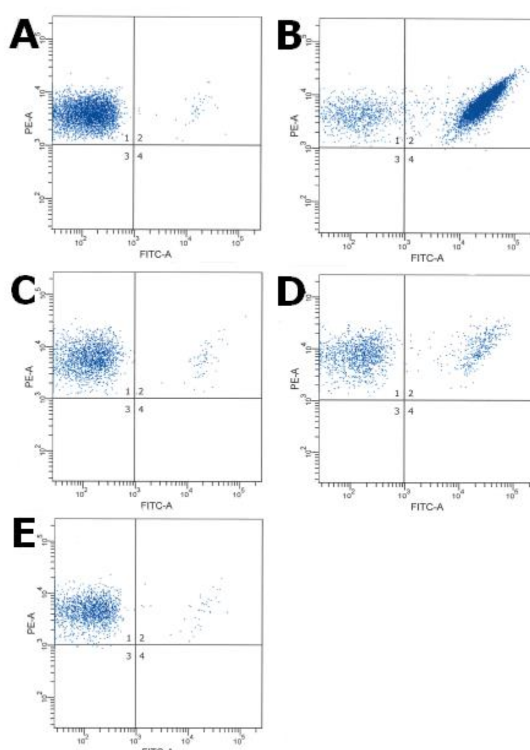
   List mode files of at least 10.000 events should be collected for FSC, SSC, and fluorescence signals for both fluorochrome conjugated antibodies with the region gated at the platelets (SSC/R-PE). Less than 10.000 events will influence the accuracy of the assay.

   Make sure to select the intermediate positive events also. These are platelet particles that are formed after activation of the platelets.



Cytogram 5. Selection of R-PE-positive platelets in SSC – R-PE plot.

5. The evaluation is done with the use of a quadrant, which is set just below the platelet marker positive population and just right to this population (see Cytogram 6 A, B, C, D and E). The percentage of activated platelets is expressed as percentage of the platelet population. Make sure both regions, the one from Cytogram 1 and the one from Cytogram 5 are both activated.



Cytogram 6. Typical figures from one set of assay samples performed with the HIT*Alert* kit and evaluated on a BD FACSCanto II **A.** unstimulated sample (I.) **B.** Ca-ionophore stimulated sample (II.) **C.** test sample without heparin (III.) **D.** test sample with heparin (IV.) and **E.** test sample with excess of heparin (V.)

Quality Control

All reagents in the HIT*Alert*™ Kit are subject to quality control.

Limitations of the Procedure

- Personnel should be trained to handle a flow cytometer and know how to interpret the data.
- The HIT*Alert*™ Kit is intended for use in combination with a flow cytometer and *not* for use with a hematology analyzer or immunofluorescence microscope.
- Accurate results with a flow cytometer depend on correct alignment and calibration of laser and detectors. The laboratory should take care of proper calibration and maintenance.
- Quality control procedures should be performed regularly as indicated in the operator's manual supplied with the flow cytometer.
- Platelet aggregation and satellitism and red blood cell auto-fluorescence may also result in unreliable results.

Warranty

Products sold here under are warranted only to conform to the quantity and contents stated on the label at the time of delivery to the customer. There are no warranties, expressed or implied, which extend beyond the description on the label of the product. IQ Products bv is not liable for property damage, personal injury, or economic loss caused by the product.

Characterization

To ensure consistently high-quality reagents, each batch of monoclonal antibody is tested for conformance with characteristics of a standard reagent.

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www.vacuette.gbo.com

Explanation of used symbols

Symbol	Meaning
(book i)	Consult instructions for use
REF	Catalogue number
Σ	Sufficient for
⚠	Caution, consult accompanying document
☀	Keep away from (sun)light
☣	Biological risks
🌡	Temperature limitation (°C)
RUO	Research Use Only
LOT	Batch code
⌛	Use by yyyy-mm-dd
🏭	Manufacturer

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