HIT*Alert*™ Kit

REF[7] IQP-396 30 tests package insert

IVD CE **In Vitro Diagnostic medical device**

Intended use

The HIT*Alert*™ Kit is used for the detection of heparin complex specific antibodies, which are capable of activation of thrombocytes (platelets) and may lead to the development of immune heparin induced thrombocytopenia.

Principle of the test

Immune heparin induced thrombocytopenia (HIT) is a distinct syndrome in which laboratory detection of the pathogenic HIT antibodies is diagnostically useful. This platelet activation assay, which detects antibodies based on their characteristic platelet-activating properties, is different from the antigen assays, which measures antibodies reactive to platelet factor 4 (PF4) – heparin complexes or PF4 – polyanion complexes. Part of the pathogenic antibodies can be specific for heparin complexes based on heparin and interleukin-8 or heparin and neutrophil-activating peptide 2 though. The HIT*Alert*™ Kit shows antibodies that recognize heparin complexes independent of the second molecule *and* it only shows those antibodies capable of inducing activation of the platelets. The antibodies that bind to the complex are able to bind to the FcγII receptor on the platelet and activate the platelet.

For the HIT*Alert*™ Kit donor platelets (PRP) are used, which are incubated in the presence of patient serum and in the presence or absence of heparin. When pathogenic antibodies are present the activation of the donor platelets is shown using a platelet activation marker. By incubating the samples with an antibody against platelets and the activation marker this reaction can be visualized using flow cytometry. For the evaluation of the stained sample a standard flow cytometer, capable of detecting FITC (FL-1) and R-PE (FL-2) fluorescence, and software are necessary.

This HIT*Alert*™ Kit should be used as a screening test. The results should always be used in conjunction with clinical findings or other serological tests.

For each suspected HIT patient a set of assay samples is tested:

I. A sample of donor PRP with heparin; to show background activation due to handling and to exclude that the PRP donor is HIT positive.

II. A sample of PRP with Ca-ionophore; to have activated thrombocytes, that can be used to set the flow cytometer.

III. A sample of donor PRP with patient serum; to show 'background' activation due to the serum.

IV. A sample of donor PRP with patient serum *and* a physiological concentration of heparin; to show activation due to the presence of heparin complex binding antibodies.

V. A sample of donor PRP with patient serum *and excess of* heparin; this sample should show a decrease in platelet activation in case of a positive sample IV, since immune complexes are disrupted due to the high concentration of heparin.

It is advised always to include a sample of a known HIT II positive patient (material NOT supplied).

HIT*Alert*™ 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 patients (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

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 patient 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 patient sample known positive for HIT. The donor most suitable will give the highest activation in sample IV (patient 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.

Processing of a fresh patient sample

- Collect venous blood from the patient into a no additive (serum) tube (for instance 454045, Greiner Vacuette), using aseptic venapuncture.
- To collect the serum, allow 30 minutes for clot forming and spin the tube 20 minutes at 1000g at RT.
- Remove the cap and collect the upper yellow fluid (serum) into an empty tube. *Stay well above the red and white cell pellet and away from the clot.*
- The serum sample should be stored at room temperature (20 to 25 °C) until processing.
- Process serum sample within 12 hours after collection. Serum can be stored for a longer period at -80 °C.

Processing of a frozen patient sample

- Frozen patient serum can also be used, for instance when the sample should be tested on a different location. It is advised to use serum that has only been frozen once.
- Store the samples on ice after thawing.
- Centrifuge the samples before use at high speed (20 minutes at 1000 g) to remove aggregates *or* filter them through a 0.2 µm centrifugal filter (for instance VWR 516-0233).

Test Procedure HIT*Alert*™ Kit

Do *not* use pre-warmed or heated (heat inactivated) patient serum.

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	Patient 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 patient sample
IV: PRP with patient sample and heparin
V: PRP with patient 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

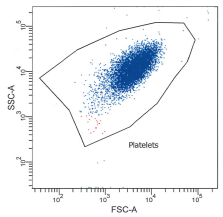
Table 2. Components to add together for adjustment of the settings of the flow cytometer. Platelets are used from assay sample II (step 4).

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.

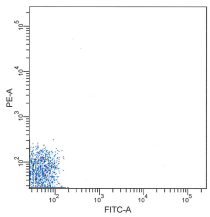
2. Adjust the voltage settings for the FSC-SSC by use of tube S1.

   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.



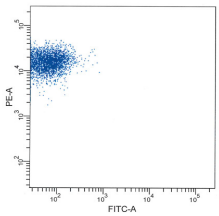
Cytogram 1. SSC (vertical) / FSC (horizontal) dot plot and region to select the platelets.

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).

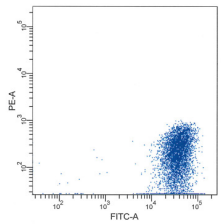


Cytogram 2. Correct adjustment of the PMT voltages for FL-2 (vertical) and FL-1 (horizontal) of the unstained sample.

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).

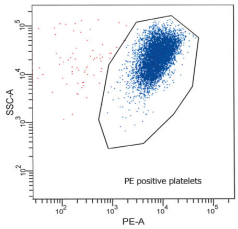


Cytogram 3. Compensation of R-PE (FL-2, vertical) signal from the FL-1 (horizontal) channel.



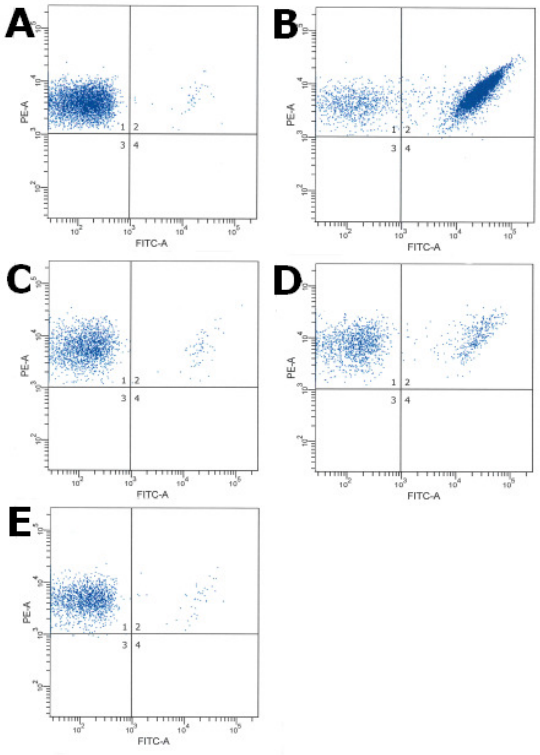
Cytogram 4. Compensation of FITC (FL-1, horizontal) signal from the FL-2 (vertical) channel.

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. 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 of a positive patient performed with the HIT*Alert* kit and evaluated on a BD FACSCanto II **A.** unstimulated sample (I.) **B.** Ca-ionophore stimulated sample (II.) **C.** patient sample without heparin (III.) **D.** patient sample with heparin (IV.) and **E.** patient sample with excess of heparin (V.)

Results

The results of the evaluation of patient blood samples are a qualitative and reliable source to determine the presence of heparin complex specific pathogenic antibodies in peripheral blood.

Interpretation

	Patient negative for HIT	Patient very likely to be positive for HIT
Sample I	< 5%	< 5%
Sample II	80-100%	80-100%
Sample III	<5%	activation (less than) half of sample IV
Sample IV	<8%	≥ 8%
Sample V	<5%	activation (less than) half of sample IV

Table 3: Typical results for a patient negative for HIT and a patient positive for HIT. A typical result for a patient positive for HIT obtained with the HIT*Alert*^{tm} Kit is shown in cytogram 6 D.

Sample I
The unstimulated platelet sample (I.) typically should have less than 1% activation marker positive platelets. When this percentage is higher than 5% the assay should be run again. Preferably with different donor PRP.

Sample II
The Ca-ionophore stimulated sample (II.) typically should have more than 80% activation marker positive platelets. A percentage lower than 80% can be due to the fact that the Ca-ionophore was not completely dissolved yet when used.

Samples III, IV and V
Sample IV, the PRP with heparin and patient sample should have an activation ≥ 8%. Typical results are shown in table 3.

There are a exceptions to table 3:

The patient sample may already contain heparin-antibody complexes from the circulation of the patient.
The number of activation marker positive events in sample IV is ≥ 8%, but there is no significant difference between sample III and IV. Sample V will show an activation that is significantly lower (typically half the activation or lower) than sample IV. The activation of the platelets is heparin dependent in that case and the result is indicative for HIT. *(See also limitations of the procedure)*

The activation is heparin independent
The number of activation marker positive events in sample IV is ≥ 8%, but there is no 'significant' difference between samples III, IV and V. Addition physiological or high concentration of heparin does not influence the percentage of activated platelets; the activation of the platelets isn't heparin depended and generally the same in all three samples.

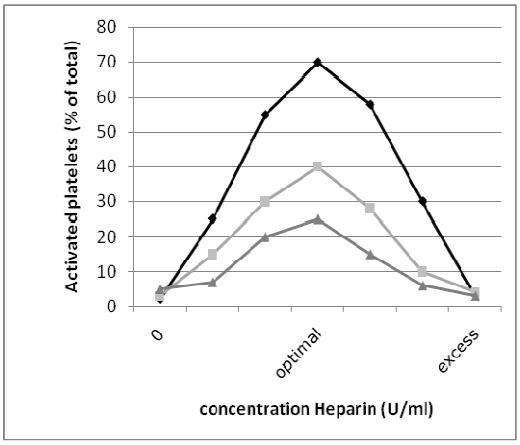


Figure 1: A typical figure for platelet activation at different heparin concentrations. The heparin in Reagent B (in sample IV) is added to the sample in the optimal concentration heparin for activation. The heparin in Reagent G is representing the excess concentration [ref 2]

This HIT*Alert*™ Kit should be used as a screening test. Results should be used in conjunction with clinical findings or other serological tests.

Quality Control

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

Limitations of the Procedure

- Personnel experienced in aseptic techniques should perform the collection of the blood sample.
- 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.
- This assay might give false positive results when PRP plasma is used from donors that have an A, B or AB positive phenotype. Platelets should be obtained from a 0-donor.
- Unreliable results can be expected with patients that (are known to) have a HAMA* response or have cold or autoagglutinins. In the clinical study only samples have been tested from persons without known clotting related diseases, like ITP and others.
- Platelet aggregation and satellitism and red blood cell auto-fluorescence may also result in unreliable results.
- Hemolitic, icteric, lipemic (of an excessive nature), bacterial contaminated specimens, specimens from patients with a myeloma or controls from other test kits have not been tested and can produce erroneous results.
- This HIT*Alert*™ Kit should be used as a screening test. Results should be used in conjunction with clinical findings and other serological tests.

* HAMA= Human Anti Mouse Antibody

Performance Characteristics

Antibody binding specificity

The antibody used in this assay has been typed in the Human Leucocyte Differentiation Antigens (HLDA) Workshop.

Clinical evaluation

During the clinical study the HIT*Alert*™ Kit has been compared to IVD approved PF4 IgG ELISA (used according to manufacturers' instructions) at two study sites. Only part of the study is represented here.
In total 195 suspected HIT samples have been tested. Besides the ELISA data, known for all samples, the final clinical diagnosis (n=149) and aggregation (n=44) data were available for part of the samples.

The Receiver Operating Characteristic (ROC) curve is good practice to compare a diagnostic assay, in this case the HIT*Alert*™ Kit, with the known clinical diagnosis of the samples.
The ROC curve shows the trade-off between the sensitivity (ability to detect the disease) and the specificity (ability to detect lack of disease). To draw the ROC curves the data had to be split in no HIT, low risk of HIT, intermediate risk of HIT and positively diagnosed HIT.

The Receiver Operating Characteristic (ROC) curve indicates that the best cut-off between positive and negative samples is 8% activation. At this level the sensitivity of the assay to discriminate between *NO* HIT and the diagnosis HIT is 78% (95% confidence interval 40% – 97%) and the specificity is 98% (95% confidence interval 91% - 100%). The same 8% cut-off was found in the combination of the samples with the low and intermediate risk 4T scores compared to HIT.

This 8% cut-off is in line with the publication of Tomer et al (2) resulting in a cut-off of 6.6% on a limited number of samples. The same paper shows a sensitivity of 95% and specificity of 100% when the flow method is compared to the SRA.

The ROC curves for the comparison of the HIT*Alert*™ Kit against *NO* HIT ('healthy'; area under the curve 0.906 (95% confidence interval 0.790 – 1.023)), low chance (area under the curve 0.884 (95% confidence interval 0.774 – 0.994)) and intermediate chance (area under the curve 0.790 (95% confidence interval 0.610 – 0.970)) are plotted in figure 2. From the respective curves it is clear that the assay discriminates the HIT cases from the negative, the low risk and the intermediate cases.

It can be concluded that the HIT*Alert*™ Kit *will positively identify the patients with the highest 4T score.*

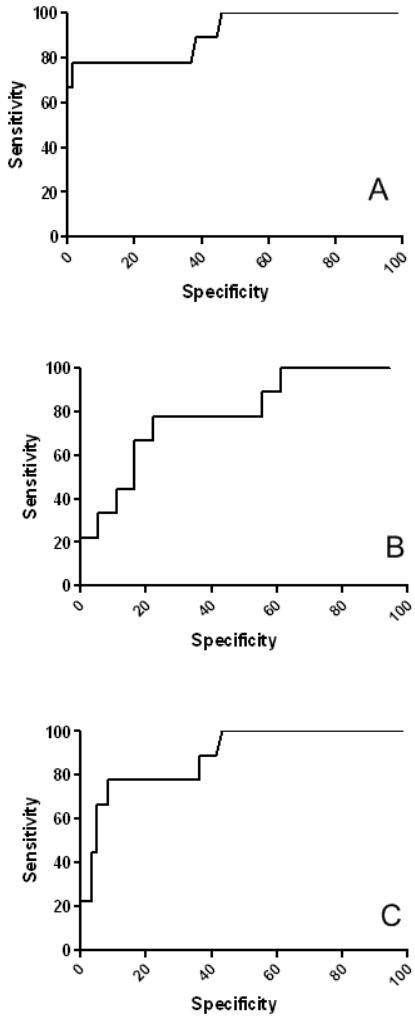


Figure 2. ROC curves of the HIT*Alert*™ Kit data of patients diagnosed positive for HIT against the patients diagnosed negative for HIT (A), against the patients diagnosed with intermediate risk of HIT (B) and against the patients with low risk of HIT (C) (definition by 4T clinical score). Areas under the curve are 0.906 (95% confidence interval 0.790 – 1.023) , 0.790 (95% confidence interval 0.610 – 0.970) and 0.884 (95% confidence interval 0.774 – 0.994) respectively.

Literature

1. Warkentin TE, and Heddle NM. Laboratory Diagnosis of Immune Heparin-induced Thrombocytopenia. Current Hematology Reports, 2003;2:148-157.
2. Tomer, A. A sensitive and specific functional flow cytometric assay for the diagnosis of heparin-induced thrombocytopenia. Br J Haematol. 1997 Sep;98(3):648-56.
3. Tomer A, Masalunga C, and Abshire TC. Determination of heparin-induced thrombocytopenia: a rapid flow cytometric assay for direct demonstration of antibody-mediated platelet activation. Am J Hematol. 1999 May;61(1):53-61.
4. Greinacher A, Juhl D, Strobel U, Wessel A, Lubenow N, Selleng K, Eichler P, and Warkentin TE. Heparin-induced thrombocytopenia: a prospective study on the incidence, platelet-activating capacity and clinical significance of antiplatelet factor 4/heparin antibodies of the IgG, IgM, and IgA classes. J Thromb Haemost. 2007; 5; 1666.
5. Tomer A. Sensitivity and specificity of laboratory tests for the diagnosis of heparin- induced thrombocytopenia. *Laboratory Hematology*. 1997;3:174-5.
6. Warkentin TE, Sheppard JI, Raschke R, and Greinacher A. Performance characteristics of a rapid assay for anti-PF4/heparin antibodies: the particle immunofiltration assay. J Thromb Haemost 2007; 5: 2308–10.
7. NEN EN ISO 15223-1 Medical devices - Symbols to be used with medical device labels, labeling and information to be supplied – Part 1: General requirements.

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.

Vacuette® is a registered trademark of Greiner Bio-One, Kremsmuenster, Austria
www.vacuette.gbo.com

Explanation of used symbols

	Consult instructions for use
REF	Catalogue number
	Sufficient for
IVD	In Vitro Diagnostic medical device
	Caution, consult accompanying document
	Keep away from (sun)light
	Biological risks
	Temperature limitation (°C)
RUO	For Research Use Only
LOT	Batch code
	Use by yyyy-mm-dd
	Manufacturer
EC REP	Authorized Representative in the European Community
CE	Conformité Européenne (European Conformity)

Contact information

IQ Products BV
www.iqproducts.nl

Rozenburglaan 13a
9727 DL Groningen
The Netherlands
T +31 (0)50 5757000
F +31 (0)50 5757002
marketing@iqproducts.nl

This product is registered as "in vitro diagnostic use" in the countries that belong to the European Community. In all other countries it should be labeled "for research use only".

This product is protected by US patent 5,763,201. IQ Products is exclusive licensee of this patent.