

AESKULISA HiT II

Ref 3290



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| Product Ref. | 3290 |
| Product Desc. | HiT II |
| Manual Rev. No. | 003 : 2013-03-01 |

Instruction Manual

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1 Intended Use

AESKULISA HiT II is a solid phase enzyme immunoassay for the quantitative and qualitative detection of IgG antibodies that cause heparin-induced thrombocytopenia type II.

2 Clinical Application and Principle of the Assay

Heparin-induced thrombocytopenia (HiT) is a severe side-effect of heparin treatment and occurs in 1-3 % of treated patients.

Two distinct types of heparin-induced thrombocytopenia can occur: HiT type I has no clinical relevance and is characterized by a transient decrease in platelet counts, which recovers after a few days even under heparin treatment. HiT type II is an immune-mediated form. The platelet counts drop more than 50 % from the baseline 5 to 14 days after starting of heparin administration. The affected patients develop antibodies recognizing neopeptides exposed by a complex of platelet factor 4 (PF4) and heparin. The antibodies found are most commonly of the IgG class. However, antibodies of the IgM and IgA class appear rarely and their pathogenic effect is still controversially discussed. HiT II autoantibodies can bind to the platelet Fcγ1a receptor and this interaction triggers platelet activation causing secretion of the coagulant thrombin. Additionally, activated platelets release PF4, which perpetuates the cycle of heparin-induced platelet activation. This effect is potentiated due to complex formation of heparin-like molecules (heparan sulfate) on the surface of endothelial cells with platelet factor 4 that can be recognized by HiT II autoantibodies. That, in turn, can induce tissue factor expression with further activation of the coagulation cascade and thrombin generation. It leads to an increased risk for new arterial and venous thromboembolic complications that can be lethal in 10-15 % of the patients.

An early diagnosis and a replacement with a suitable alternative anticoagulant can clearly minimize the complication rate.

Principle of the test

Serum samples diluted 1:101 are incubated in the microplates coated with the specific antigen. Patient's antibodies, if present in the specimen, bind to the antigen. The unbound fraction is washed off in the following step. Afterwards anti-human immunoglobulins conjugated to horseradish peroxidase (conjugate) are incubated and react with the antigen-antibody complex of the samples in the microplates. Unbound conjugate is washed off in the following step. Addition of TMB-substrate generates an enzymatic colorimetric (blue) reaction, which is stopped by diluted acid (color changes to yellow). The intensity of color formation from the chromogen is a function of the amount of conjugate bound to the antigen-antibody complex and this is proportional to the initial concentration of the respective antibodies in the patient sample.

3 Kit Contents

| TO BE RECONSTITUTED | | | | |
|---|--------------------|-----------|----------------|--|
| Item | Quantity | Cap color | Solution color | Description / Contents |
| Sample Buffer (5x) | 1 x 20ml | White | Yellow | 5 x concentrated Tris, sodium chloride (NaCl), bovine serum albumin (BSA), sodium azide < 0.1% (preservative) |
| Wash Buffer (50x) | 1 x 20ml | White | Green | 50 x concentrated Tris, NaCl, Tween 20, sodium azide < 0.1% (preservative) |
| READY TO USE | | | | |
| Item | Quantity | Cap color | Solution color | Description / Contents |
| Negative Control | 1 x 1.5ml | Green | Colorless | Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative) |
| Positive Control | 1 x 1.5ml | Red | Yellow | Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative) |
| Cut-off Calibrator | 1 x 1.5ml | Blue | Yellow | Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative) |
| Calibrators | 6 x 1.5ml | White | Yellow * | Concentration of each calibrator: 0, 3, 10, 30, 100, 300 U/ml. Human serum (diluted), bovine serum albumin (BSA), sodium azide < 0.1% (preservative) |
| Conjugate, IgG | 1 x 15ml | Blue | Blue | Containing: Anti-human immunoglobulins conjugated to horseradish peroxidase, bovine serum albumin (BSA) |
| TMB Substrate | 1 x 15ml | Black | Colorless | Stabilized tetramethylbenzidine and hydrogen peroxide (TMB/H ₂ O ₂) |
| Stop Solution | 1 x 15ml | White | Colorless | 1M Hydrochloric Acid |
| Microtiter plate | 12 x 8 well strips | N/A | N/A | With breakaway microwells. Refer to paragraph 1 for coating. |
| * Color increasing with concentration | | | | |
| MATERIALS REQUIRED, BUT NOT PROVIDED | | | | |
| Microtiter plate reader 450 nm reading filter and recommended 620 nm reference filter (600-690 nm). Glass ware (cylinder 100-1000ml), test tubes for dilutions. Vortex mixer, precision pipettes (10, 100, 200, 500, 1000 µl) or adjustable multipipette (100-1000µl). Microplate washing device (300 µl repeating or multichannel pipette or automated system), adsorbent paper. Our tests are designed to be used with purified water according to the definition of the United States Pharmacopeia (USP 26 - NF 21) and the European Pharmacopeia (Eur.Ph. 4th ed.). | | | | |

4 Storage and Shelf Life

Store all reagents and the microplate at 2-8°C/35-46°F, in their original containers. Once prepared, reconstituted solutions are stable at 2-8°C/35-46°F for at least 1 month. Reagents and the microplate shall be used within the expiry date indicated on each component, only. Avoid intense exposure of TMB solution to light. Store microplates in designated foil, including the desiccant, and seal tightly.



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5 Precautions of Use

5.1 Health hazard data

THIS PRODUCT IS FOR IN VITRO DIAGNOSTIC USE ONLY. Thus, only staff trained and specially advised in methods of in vitro diagnostics may perform the kit. Although this product is not considered particularly toxic or dangerous in conditions of the intended use, refer to the following for maximum safety:

Recommendations and precautions

This kit contains potentially hazardous components. Though kit reagents are not classified being irritant to eyes and skin we recommend to avoid contact with eyes and skin and wear disposable gloves.

WARNING ! Calibrators, Controls and Buffers contain sodium azide (NaN₃) as a preservative. NaN₃ may be toxic if ingested or adsorbed by skin or eyes. NaN₃ may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up. Please refer to decontamination procedures as outlined by CDC or other local/national guidelines.

Do not smoke, eat or drink when manipulating the kit. Do not pipette by mouth.

All human source material used for some reagents of this kit (controls, standards e.g.) has been tested by approved methods and found negative for HbsAg, Hepatitis C and HIV 1. However, no test can guarantee the absence of viral agents in such material completely. Thus handle kit controls, standards and patient samples as if capable of transmitting infectious diseases and according to national requirements.

The kit contains material of animal origin as stated in the table of contents, handle according to national requirements.

5.2 General directions for use

In case that the product information, including the labeling, is defective or incorrect please contact the manufacturer or the supplier of the test kit.

Do not mix or substitute Controls, Calibrators, Conjugates or microplates from different lot numbers. This may lead to variations in the results.

Allow all components to reach room temperature (20-32 °C/68-89.6 °F) before use, mix well and follow the recommended incubation scheme for an optimum performance of the test.

Incubation: We recommend test performance at 30 °C/86 °F for automated systems.

Never expose components to higher temperature than 37 °C/ 98.6 °F.

Always pipette substrate solution with brand new tips only. Protect this reagent from light. Never pipette conjugate with tips used with other reagents prior.

A definite clinical diagnosis should not be based on the results of the performed test only, but should be made by the physician after all clinical and laboratory findings have been evaluated. The diagnosis is to be verified using different diagnostic methods.



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6 Sample Collection, Handling and Storage

Use preferentially serum samples or citrated plasma samples freshly collected with 3.2% or 3.8% sodium citrate as an anticoagulant. Blood withdrawal must follow national requirements. Do not use icteric, lipemic, hemolysed or bacterially contaminated samples. Blood samples should be collected in clean, dry and empty tubes. After centrifugation, the plasma samples should be used immediately, otherwise stored tightly closed at 2-8 °C/35-46 °F up to eight hours, or frozen at -20 °C/-4 °F for longer periods.

Do not use samples anticoagulated with heparin in this assay!

7 Assay Procedure

7.1 Preparations prior to starting

Dilute concentrated reagents:

Dilute the concentrated sample buffer 1:5 with distilled water (e.g. 20 ml plus 80 ml).

Dilute the concentrated wash buffer 1:50 with distilled water (e.g. 20 ml plus 980 ml).

To avoid mistakes we suggest to mark the cap of the different calibrators.

Samples:

Dilute serum samples 1:101 with sample buffer (1x)

e.g. 1000 µl sample buffer (1x) + 10 µl serum. Mix well !

Washing:

Prepare 20 ml of diluted wash buffer (1x) per 8 wells or 200 ml for 96 wells

e.g. 4 ml concentrate plus 196 ml distilled water.

Automated washing:

Consider excess volumes required for setting up the instrument and dead volume of robot pipette.

Manual washing:

Discard liquid from wells by inverting the plate. Knock the microwell frame with wells downside vigorously on clean adsorbent paper. Pipette 300 µl of diluted wash buffer into each well, wait for 20 seconds. Repeat the whole procedure twice again.

Microplates:

Calculate the number of wells required for the test. Remove unused wells from the frame, replace and store in the provided plastic bag, together with desiccant, seal tightly (2-8 °C/35-46 °F).

7.2 Pipetting Scheme

We suggest pipetting calibrators, controls and samples as follows:

| For <i>QUANTITATIVE</i> interpretation | | | | | For <i>QUALITATIVE</i> interpretation | | | | |
|--|-------|-------|-----|------|---------------------------------------|----|-----|---|------|
| | 1 | 2 | 3 | 4... | | 1 | 2 | 3 | 4... |
| A | Cal A | Cal E | P1 | | A | NC | P2 | | |
| B | Cal A | Cal E | P1 | | B | NC | P2 | | |
| C | Cal B | Cal F | P2 | | C | CC | P3 | | |
| D | Cal B | Cal F | P2 | | D | CC | P3 | | |
| E | Cal C | PC | P3 | | E | PC | ... | | |
| F | Cal C | PC | P3 | | F | PC | ... | | |
| G | Cal D | NC | ... | | G | P1 | ... | | |
| H | Cal D | NC | ... | | H | P1 | ... | | |

Cal A: calibrator A

Cal B: calibrator B

Cal C: calibrator C

Cal D: calibrator D

Cal E: calibrator E

Cal F: calibrator F

PC: positive control

NC: negative control



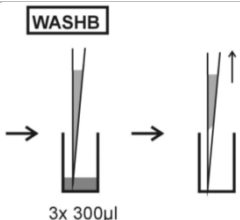
CC: cut-off calibrator

P1: patient 1


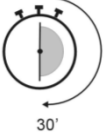
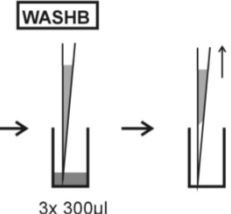
P2: patient 2

P3: patient 3



7.3 Test Steps

| Step | Description |
|-------------------------------|--|
| 1. | Ensure preparations from step 7.1 above have been carried out prior to pipetting. Allow all components to reach room temperature (20-32°C/68-89.6°F) before use, mix well and follow the recommended incubation scheme. |
| 2. | Use the following steps in accordance with quantitative/ qualitative interpretation results desired: |
| CONTROLS & SAMPLES | |
| 3. |  <p>Pipette into the designated wells as described in chapter 7.2, 100 µl of either:</p> <ol style="list-style-type: none"> Calibrators (Cal A to Cal F) for <i>QUANTITATIVE</i> or Cut-off Calibrator (CC) for <i>QUALITATIVE</i> interpretation <p>and 100 µl of each of the following:</p> <ul style="list-style-type: none"> Negative control (NC) and Positive control (PC) Patients diluted serum (P1, P2...) |
| 4. |  <p>Incubate for 30 minutes at 20-32°C/68-89.6°F.</p> |
| 5. |  <p>Wash 3x with 300 µl washing buffer (diluted 1:50).</p> |

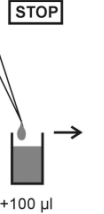

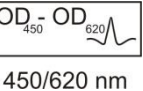
CONJUGATE

| | | |
|----|---|--|
| 6. |  | <p>Pipette 100 μl conjugate into each well.</p> |
| 7. |  | <p>Incubate for 30 minutes at 20-32 $^{\circ}$C/68-89.6 $^{\circ}$F.</p> |
| 8. |  | <p>Wash 3x with 300 μl washing buffer (diluted 1:50).</p> |

SUBSTRATE

| | | |
|-----|---|--|
| 9. |  | <p>Pipette 100 μl TMB substrate into each well.</p> |
| 10. |  | <p>Incubate for 30 minutes at 20-32 $^{\circ}$C/68-89.6 $^{\circ}$F, protected from intense light.</p> |

STOP

| | | |
|-----|---|---|
| 11. |  | <p>Pipette 100 μl stop solution into each well, using the same order as pipetting the substrate.</p> |
| 12. |  | <p>Incubate 5 minutes minimum.</p> |
| 13. | | <p>Agitate plate carefully for 5 sec.</p> |
| 14. |  | <p>Read absorbance at 450 nm within 30 minutes. It is recommended to use the difference of absorbance at 450 and 620 nm).</p> |



8 Quantitative and Qualitative Interpretation

For **quantitative interpretation** establish the standard curve by plotting the **optical density (OD) of each calibrator (y-axis)** with respect to the corresponding concentration values in U/ml (x-axis). For best results we recommend log/lin coordinates and 4-Parameter Fit. From the OD of each sample, read the corresponding antibody concentrations expressed in U/ml.

| Normal Range | Equivocal Range | Positive Results |
|--------------|-----------------|------------------|
| < 12 U/ml | 12 - 18 U/ml | >18 U/ml |

Example of a standard curve

Do NOT use this example for interpreting patient's result

| Calibrators IgG | OD 450/620 nm | CV % (Variation) |
|-----------------|---------------|------------------|
| 0 U/ml | 0.025 | 0.0 |
| 3 U/ml | 0.139 | 3.5 |
| 10 U/ml | 0.283 | 4.3 |
| 30 U/ml | 0.598 | 4.0 |
| 100 U/ml | 1.224 | 3.6 |
| 300 U/ml | 2.123 | 2.8 |

Example of calculation

| Patient | Replicate (OD) | Mean (OD) | Result (U/ml) |
|---------|----------------|-----------|---------------|
| P 01 | 0.793/0.801 | 0.797 | 47.7 |
| P 02 | 0.308/0.333 | 0.321 | 12.1 |

Samples above the highest calibrator range should be reported as >Max. They should be diluted as appropriate and re-assayed. Samples below calibrator range should be reported as < Min.

For lot specific data, see enclosed quality control leaflet. Medical laboratories might perform an in-house quality control by using own controls and/or internal pooled sera, as foreseen by national regulations.

Each laboratory should establish its own normal range based upon its own techniques, controls, equipment and patient population according to their own established procedures.

In case that the values of the controls do not meet the criteria the test is invalid and has to be repeated.

The following technical issues should be verified: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, photometer, incubation conditions and washing methods.

If the items tested show aberrant values or any kind of deviation or that the validation criteria are not met without explicable cause please contact the manufacturer or the supplier of the test kit.

For **qualitative interpretation** read the optical density of the cut-off calibrator and the patient samples. Compare patient's OD with the OD of the cut-off calibrator. For qualitative interpretation we recommend to consider sera within a range of 20% around the cut-off value as equivocal. All samples with higher ODs are considered positive, samples with lower ODs are considered negative.

Negative: OD patient < 0.8 x OD cut-off
Equivocal: 0.8 x OD cut-off ≤ OD patient ≤ 1.2 x OD cut-off
Positive: OD patient > 1.2 x OD cut-off



9 Technical Data

| | |
|---------------------------|---|
| Sample material: | Serum or citrated plasma |
| Sample volume: | 10 µl of sample diluted 1:101 with 1x sample buffer |
| Total incubation time: | 90 minutes at 20-32 °C/68-89.6 °F |
| Calibration range: | 0-300 U/ml |
| Analytical sensitivity: | 1.0 U/ml |
| Storage: | at 2-8 °C/35-46 °F use original vials only. |
| Number of determinations: | 96 tests |

10 Performance Data

10.1 Analytical sensitivity

The detection limit determined by 8fold testing of at least 8 negative serum samples and a 60fold determination of sample buffer amount to 1.0 U/ml.

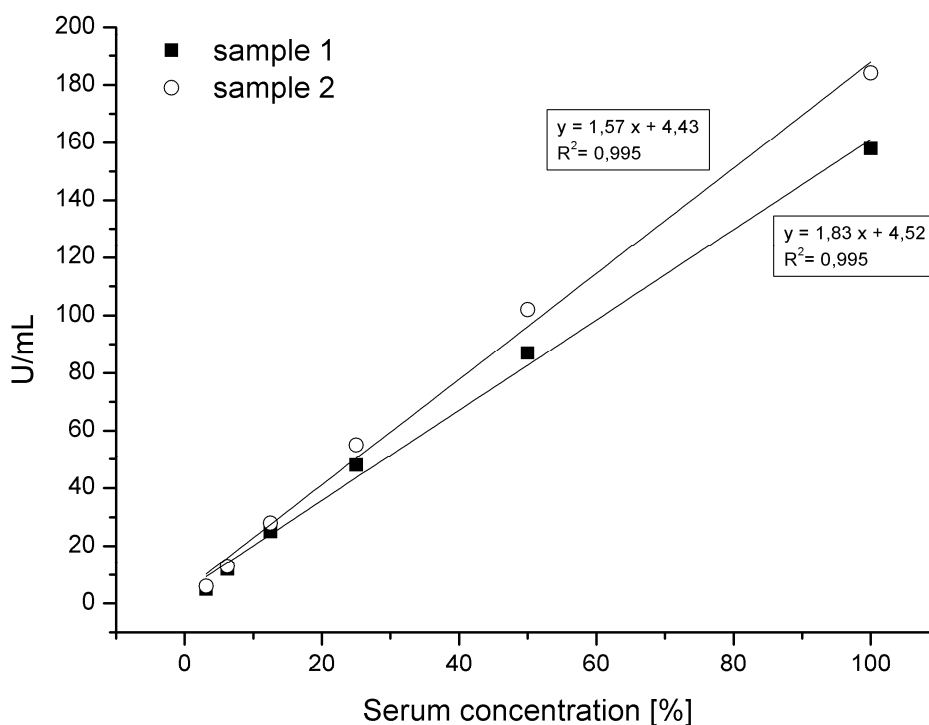
10.2 Specificity and sensitivity

By the use of clinically defined serum samples with known immune status a diagnostic sensitivity of 91 % and a specificity of 97 % was determined for the AESKULISA HiT II.

10.3 Linearity

To determine the linearity of the AESKULISA HiT II, serial dilutions of sera were measured. The obtained results were compared to the expected ones, which were calculated by the quotient of measured value of the next higher concentration and the dilution factor 2. Recovery is the percentage of the measured value to the expected one.

| Sample No. | Dilution Factor | Serum concentration | Measured (U/ml) | Expected (U/ml) | Recovery (%) |
|------------|-----------------|---------------------|-----------------|-----------------|--------------|
| 1 | 1 / 100 | 100% | 184 | 184 | 100.0 |
| | 1 / 200 | 50% | 101.5 | 91.8 | 110.6 |
| | 1 / 400 | 25% | 55.3 | 50.8 | 108.9 |
| | 1 / 800 | 12,5% | 28.4 | 27.6 | 102.9 |
| | 1 / 1600 | 6,25% | 13.4 | 14.2 | 94.4 |
| 2 | 1 / 100 | 100% | 158 | 158 | 100.0 |
| | 1 / 200 | 50% | 86.9 | 78.9 | 110.1 |
| | 1 / 400 | 25% | 48 | 43.5 | 110.3 |
| | 1 / 800 | 12,5% | 24.7 | 24 | 102.9 |
| | 1 / 1600 | 6,25% | 11.6 | 12.4 | 93.5 |



Tested serum samples show a linear correlation between sample dilution and antibody concentration. However, due to the heterogeneous nature of human autoantibodies there might be samples possessing a non-linear behavior.

10.4 Precision

To determine the precision of the assay, the variability (intra and inter-assay) was assessed by examining its reproducibility on serum samples selected to represent the standard curve.

| Intra-assay | | |
|-------------|-------------|--------|
| Sample No. | Mean (U/ml) | CV (%) |
| 1 | 13 | 5 |
| 2 | 22 | 5 |
| 3 | 63 | 4 |
| 4 | 192 | 7 |

| Inter-assay | | |
|-------------|-------------|--------|
| Sample No. | Mean (U/ml) | CV (%) |
| 1 | 13 | 8 |
| 2 | 22 | 6 |
| 3 | 63 | 6 |
| 4 | 192 | 11 |

10.5 Calibration

Due to the lack of international reference calibration this assay is calibrated in arbitrary units (U/ml).



11 Literature

Warkentin T.E. (2005). New approaches to the diagnosis of heparin induced thrombocytopenia. *Chest* 127: 35-45.

Franchini M. (2005). Heparin induced thrombocytopenia: an update. *Thrombosis Journal* 3: 14.

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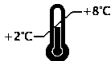












Lindhoff-Last E., Gerdson F., Ackermann H., Bauersachs R. (2001). Determination of heparin-platelet factor 4-IgG antibodies improves diagnosis of heparin induced thrombocytopenia. *British Journal of Haematology* 113: 886-890.

Warkentin T.E., Chong B.H., Greinacher A. (1998). Heparin induced thrombocytopenia: Towards consensus. *Thrombosis and Haemostasis* 79: 1-7

Ziporen L., Li Z.Q., Park K.S., Sabnekar P., Liu W.Y., Arepally G., Shoenfeld Y., Kieber-Emmons T., Cines D.B., Poncz M. (1998). Defining an antigenic epitope on platelet factor 4 associated with heparin-induced thrombocytopenia. *Blood* 92, 9: 3250-3259.

Greinacher A., Poetsch B., Amiral J., Dummel V., Eichner A., Mueller-Eckhardt C. (1994). Heparin-associated thrombocytopenia: isolation of the antibody and characterization of a multimolecular PF4-heparin complex as the major antigen. *Thrombosis and Haemostasis* 71: 247-251.

Chong B.H., Fawaz I., Chesterman C.N., Berndt M.C. (1989). Heparin induced thrombocytopenia: mechanism of interaction of the heparin-dependent antibody with platelets. *British Journal of Haematology* 73: 235-240.

| | | |
|---|--|---|
|  | - Diagnosi in vitro - Pour diagnostic in vitro - In Vitro Diagnostikum - Para uso Diagnóstico in vitro | - For in vitro diagnostic use - Para uso diagnóstico in vitro - In Vitro Διαγνωστικό μέσο |
|  | ~ Numero d'ordine ~ Référence Catalogue ~ Bestellnummer ~ Número de catálogo | ~ Catalogue number ~ Numéro de catálogo ~ Αριθμός παραγγελίας |
|  | ~ Descrizione lotto ~ Lot ~ Chargen Bezeichnung ~ Lote | ~ Lot ~ Lote ~ Χαρακτηρισμός παρτίδας |
|  | ~ Conformità europea ~ Déclaration CE de Conformité ~ Europäische Konformität ~ Declaração CE de Conformidade | ~ EC Declaration of Conformity ~ Declaración CE de Conformidad ~ Ευρωπαϊκή συμφωνία |
|  | ~ 96 determinazioni ~ 96 tests ~ 96 Bestimmungen ~ 96 Testes | ~ 96 tests ~ 96 pruebas ~ 96 προσδιορισμοί |
|  | ~ Rispettare le istruzioni per l'uso ~ Voir les instructions d'utilisation ~ Gebrauchsanweisung beachten ~ Ver as instruçoes de uso | ~ See instructions for use ~ Ver las instrucciones de uso ~ Λάβετε υπόψη τις οδηγίες χρήσης |
|  | ~ Da utilizzarsi entro ~ Utilise avant le ~ Verwendbar bis ~ Utilizar antes de | ~ Use by ~ Utilizar antes de ~ Χρήση μέχρι |
|  | ~ Conservare a 2-8 °C ~ Conserver à 2-8 °C ~ Lagerung bei 2-8 °C ~ Conservar entre 2-8 °C | ~ Store at 2-8 °C (35-46 °F) ~ Conservar a 2-8 °C ~ Φυλάσσεται στους 2-8 °C |
|  | ~ Prodotto da ~ Fabriqué par ~ Hergestellt von ~ Fabricado por | ~ Manufactured by ~ Fabricado por ~ Κατασκευάζεται από |
|  | ~ Calibratore cut-off ~ Etalon Seuil ~ Grenzwert Kalibrator ~ Calibrador de cut-off | ~ Cut off Calibrator ~ Calibrador de cut-off ~ Οριακός ορός Αντιδραστήριο βαθμονόμησης |
|  | ~ Controllo positivo ~ Contrôle Positif ~ Positiv Kontrolle ~ Controllo positivo | ~ Positive Control ~ Control Positivo ~ Θετικός ορός ελέγχου |
|  | ~ Controllo negativo ~ Contrôle Négatif ~ Negativ Kontrolle ~ Controllo negativo | ~ Negative Control ~ Control Negativo ~ Αρνητικός ορός ελέγχου |
|  | ~ Calibratore ~ Etalon ~ Kalibrator ~ Calibrador | ~ Calibrator ~ Calibrador ~ Αντιδραστήριο βαθμονόμησης |
|  | ~ Recupero ~ Corrélation ~ Wiederfindung ~ Recuperação | ~ Recovery ~ Recuperado ~ Ανάκτηση |
|  | ~ Coniugato ~ Conjugé ~ Konjugat ~ Conjugado | ~ Conjugate ~ Conjugado ~ Σύζευγμα |
|  | ~ Micropiastra rivestita ~ Microplaque sensibilisée ~ Beschichtete Mikrotiterplatte ~ Microplaca revestida | ~ Coated microtiter plate ~ Microplaca sensibilizada ~ Επικαλυμμένη μικροπλάκα |
|  | ~ Tampone di lavaggio ~ Tampon de Lavage ~ Waschpuffer ~ Solução de lavagem | ~ Wash buffer ~ Solución de lavado ~ Ρυθμιστικό διάλυμα πλύσης |
|  | ~ Tampone substrato ~ Substrat ~ Substratpuffer ~ Substrato | ~ Substrate buffer ~ Tampón sustrato ~ Ρυθμιστικό διάλυμα υποστρώματος |
|  | ~ Reagente bloccante ~ Solution d'Arrêt ~ Stopreagenz ~ Solução de paragem | ~ Stop solution ~ Solución de parada ~ Αντιδραστήριο διακοπής αντίδρασης |
|  | ~ Tampone campione ~ Tampon Echantillons ~ Probenpuffer ~ Diluente de amostra | ~ Sample buffer ~ Tampón Muestras ~ Ρυθμιστικό διάλυμα δειγμάτων |