DRG® Anti-Human Hsp70 (lgG/A/M) ELISA (EIA-4470)

REVISED 23 DEC. 2008 (VERS. 2.0) **RUO**

1 INTRODUCTION

1.1 ASSAY DESIGN

The DRG Anti-Human Hsp70 (lgG/A/M) ELISA (enzyme-linked immunosorbent assay) Kit provides a method to detect and quantitate antibodies to human Hsp70 in human serum samples.

This assay allows for reproducible, accurate and precise determination of IgG, IgA and IgM antibodies to human Hsp70.

The Anti-Human Hsp70 (lgG/A/M) ELISA Kit uses recombinant human Hsp70 pre-coated to the wells of the Rec. Human Hsp70 Immunoassay Plate to capture anti-human Hsp70 antibodies present in human serum. The captured anti-human Hsp70 antibodies are detected with a hydrogen peroxidase conjugated goat polyclonal antibody specific for human IgG, IgAand IgM molecules.

The assay is developed with tetramethylbenzidine substrate producing a blue color in proportion to the amount of captured anti-human Hsp70 antibodies. The color development is stopped with acid stop solution which converts the endpoint color to yellow. The intensity of the color is measured in a microplate reader at 450nm.

1.2 SCIENTIFIC OVERVIEW

Traditional methods for detection and quantitation of antihuman Hsp70 antibody were accomplished by using pre-screened serum samples with a high level of anti-human Hsp70 antibody. These samples were assigned a concentration of 1000 arbitrary units/mL (Aunits/mL) and were used to generate standard dose-response curves from which antibody levels in test samples were determined.

DRG Anti-Human Hsp70 (IgG/A/M) ELISA kit uses a calibrated standard of anti-human Hsp70 (IgG/A/M) antibodies isolated from pooled human sera to generate a standard curve. The kit provides researchers with a rapid, reliable and standardized method to measure the levels of anti-human Hsp70 antibody levels in human serum samples by interpolating absorbance readings from the standard curve. This kit has the potential of expanding our knowledge of the role of anti-human Hsp70 antibodies in the normal population and as a diagnostic tool to evaluate and monitor a variety of diseases.

The inducible heat shock protein, Hsp70 (Hsp72) is part of the Hsp70 family which contains a number of highly related protein isoforms ranging in size from 66 kDa to 78 kDa. These proteins include both cognate members which are found within major intracellular compartments and highly inducible isoforms which appear to be predominantly cytoplasmic or nuclear in distribution. Members of the Hsp70 family are molecular chaperones that are involved in many cellular functions such as protein folding, transport, maturation and degradation, exerting their function in an ATP-dependent manner. The molecular chaperones of the Hsp70 family recognize and bind to nascent polypeptide chains as well as partially folded intermediates of proteins preventing their aggregation and misfolding [1]. Inducible Hsp70 is typically regarded as an intracellular protein. However studies have shown the presence of soluble Hsp70 and anti-Hsp70 antibodies in the peripheral circulation of normal individuals [2, 3] and in various disease states in the following instances.

The presence of circulating anti-Hsp70 antibodies was detected more frequently in smokers versus non-smokers [4]. In patients with Graves' disease higher anti-Hsp70 antibody levels were measured compared to controls [4]. Patients with uveitis were found to have circulating levels of anti-Hsp70 antibody and these levels may reflect the extent of disease involvement within the eye [5]. Antibodies against various heat shock proteins including Hsp70 were detected in sera of patients with dilated cardiomyopathy as compared to healthy controls [6]. Acorrelation between anti-Hsp70 antibodies and different types of vascular diseases exists suggesting that Hsp70 might be involved in the pathogenesis and propagation of atherosclerosis [7]. There is a possible association of plasma anti-Hsp70 antibody levels with hypertension and harsh working conditions [8]. Patients with severe heat-induced symptoms showed significantly higher anti-Hsp70 antibody levels [9]. Antibodies to Hsp70 have been associated with graft-versus-host disease in peripheral blood stem cell transplant recipients [10]. Hsp70 has been implicated as a potential autoantigen in multiple sclerosis and enhanced expression of several heat shock proteins including Hsp70 in myelin may subsequently present as additional immune targets involved in the progression of this disease [11, 12, 13]. Anti-Hsp70 antibodies may be involved in the pathogenesis of schizophrenia and especially high anti-Hsp70 titers were found in never-medicated patients [14, 15]. Formation of Hsp70-antibody complexes in the placenta correlated with anti-Hsp70 antibody levels in sera and these complexes may contribute to the induction of preterm birth. Women sensitized to these antibodies may be at increased risk for adverse pregnancy outcomes [16]. Hsp70 and anti-Hsp70 antibodies may have diagnostic and prognostic value for different gynecologic malignancies [17, 18].

The ubiquitous nature of Hsp70 and the high degree of sequence homology between mammals and bacterial heat shock proteins may provide a link between infection and autoimmunity. Further studies are required to evaluate the physiological and immunological relevance of circulating Hsp70 and anti-Hsp70 antibodies and their interaction in autoimmune and inflammatory conditions [19].

1.3 ASSAY PROCEDURE SUMMARY

1. Allow the Rec. Human Hsp70 Immunoassay Plate, 20X Wash Buffer, Sample Diluent, TMB Substrate and Stop Solution to warm to room temperature at least 30 minutes prior to opening.
2. Centrifuge Anti-Human Hsp70 Standard before removing cap.
   Caution: This component is derived from human serum. Treat as biohazard.
3. Dilute Anti-Human Hsp70 Standard and samples in Sample Diluent.
4. Add 100 µLprepared standards and samples in duplicate to wells of Rec. Human Hsp70 Immunoassay Plate. Cover immunoassay plate.
5. Incubate plate at room temperature for 2 hours.
6. Wash wells 4X with 1X Wash Buffer.
7. Add 100 µL Anti-Human GAM-HRP Conjugate to each well. Cover immunoassay plate.
8. Incubate plate at room temperature for 1 hour.
9. Wash wells 4X with 1X Wash Buffer.
10. Add 100µL TMB Substrate to each well.
11. Incubate at room temperature for 15 minutes.
12. Add 100 µL Stop Solution to each well.
13. Measure absorbance at 450 nm, or 450 nm with a correction at 540 or 570 nm.
14. Plot the anti-human Hsp70 (IgG/A/M) standard curve and calculate anti-human Hsp70 sample concentrations.

2 MATERIALS

2.1 PRECAUTIONS

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

- **Caution!** The Anti-Human Hsp70 Standard is derived from human serum. Treat as biohazard.
  Care should be taken in handling this material to minimize possible contamination with infectious agents present in human serum. The serum from which this product is derived was tested by an FDA approved method and found negative for HIV-1, HIV-2, HIV p24 Ag and non reactive to HbsAg, HVC-3 and STS. No known test method can offer complete assurance that Hepatitis B virus, Hepatitis C virus, HIV-1, HIV-2 or other infectious agents are absent.
- The Stop Solution is a solution of hydrochloric acid.
  This solution is corrosive; please use caution when handling.
- The activity of the Anti-Human GAM-HRP Conjugate is affected by nucleophiles such as azide, cyanide and hydroxylamine.

Please read the complete kit insert before performing this assay.

2.2 MATERIALS PROVIDED

The Anti-Human Hsp70 (lgG/A/M) ELISA Kit contains the following components in sufficient quantities for 96 wells. These reagents are sufficient to assay one standard curve and 41 samples in duplicate or two standard curves and 34 samples in duplicate.

COMPONENT	SIZE	DESCRIPTION
Immunoassay Plate	96 well plate	12 x 8 removable strips and frame. Pre-coated plate with recombinant human Hsp70 antigen
Standard	120 µL	Human serum containing antihuman Hsp70 IgG, IgA, IgM antibodies
Sample Diluent	100 mL	Buffer to dilute standards and samples
20X Wash Buffer	100 mL	Concentrated solution of buffer and surfactant
Anti-Human GAM-HRP Conjugate	10 mL	Horseradish Peroxidase conjugated polyclonal antibody specific for human IgA, IgG, IgM antibodies
TMB Substrate Solution	10 mL	Stabilized tetramethylbenzidine substrate
Stop Solution	10 mL	Acid solution to stop color reaction

2.3 STORAGE OF MATERIALS

All reagents are stable as supplied at 4°C, **except the Anti-Human Hsp70 Standard**, which should be stored at 20°C.

Unused wells of the Rec. Human Hsp70 Immunoassay Plate should be resealed in the foil pouch provided and stored at 4°C until the kits expiry date.

2.4 MATERIALS REQUIRED BUT NOT PROVIDED

- Deionized or distilled water
- Precision pipettors capable of accurately delivering 1 to 1000 µL
- Disposable pipette tips
- 5, 10, 25 mL pipettes for reagent preparation
- 1L graduated cylinder
- Squirt bottle, manifold dispenser, or automated microtitre plate washer
- Disposable polypropylene tubes
- Microtiter plate reader capable of measuring absorbance at 450 nm

3 PERFORMING THE ASSAY

3.1 CRITICAL ASSAY PARAMETERS AND NOTES

- DRG Anti-Human Hsp70 (lgG/A/M) ELISA Kit contains a pre-coated microtiter plate (Rec. Human Hsp70 Immunoassay Plate) with removable wells to allow assaying on two separate occasions.
- Run both standards and samples in duplicate.
- Include a standard curve each time the assay is performed.
- The following kit components should be allowed to warm to room temperature at least 30 minutes prior to use: Rec. Human Hsp70 Immunoassay Plate, 20X Wash Buffer, Sample Diluent, TMB Substrate, Stop Solution.
- Absorbance is a function of the incubation time. Therefore, prior to starting the assay it is recommended that all reagents are ready to use and all required strip-wells secured in the microtiter frame. This will ensure equal elapsed time for each pipetting step, without interruption.
- Mix all reagents and samples gently, yet thoroughly, prior to use. Avoid foaming of reagents.
- To avoid cross contamination, change disposable pipette tips between the addition of each standard, samples, and reagents. Use separate reagent troughs/reservoirs for each reagent.
- This assay requires pipetting of small volumes. To minimize imprecision caused by pipetting, ensure that pipettes are calibrated.
- Consistent, thorough washing of each well is critical. If using an automatic washer, check washing head before use. If washing manually, ensure all wells are completely filled at each wash. Air bubbles should be avoided.
- Exercise appropriate laboratory safety precautions when performing this assay.

NOTE: The components in each kit lot numbers have been quality assured and warranted in this specific combination only; please do not mix them with components from other kit lot numbers.

3.2 SAMPLE PREPARATION

1. COLLECTION OF SERUM

a. Collect whole blood using a serum separator tube.
b. Allow samples to clot at room temperature for 3 minutes.
c. Centrifuge at approximately 1000 x g for 10 minutes, taking precautions to avoid hemolysis.
d. Remove serum. Transfer the serum to a labeled polypropylene tube. The serum collected is now ready for analysis using the Anti-Human Hsp70 (lgG/A/M) ELISA Kit.
e. Alternatively, the serum sample can be frozen at -20°C and assayed at a later date. It is recommended that the serum be aliquoted to convenient volumes prior to storing at -20°C to avoid multiple freeze thaw cycles.

2. DILUTION OF SAMPLES

Serum samples should be prepared as described in #1 above.

Serum can be diluted 1:1000 (v/v) in Sample Diluent by a two step serial dilution (1:10 followed by a 1:100 dilution). This is a suggested starting dilution only. Additional dilutions may be necessary to ensure that sample values are within the range of the standard curve. Users must determine the optimal sample dilutions for their particular experiments.

a. Dilute prepared samples in Sample Diluent. Prepare at least 250 µLof diluted sample to permit assaying in duplicate.
b. Mix thoroughly.
c. Samples are now ready to be used in the Assay Procedure. Samples may be left at room temperature while reagents are being prepared.

3.3 REAGENT PREPARATION

NOTE: *All reagents should be freshly prepared prior to use. Once prepared, reagents should be kept at room temperature for the duration of the assay.*

NOTE: *The preparation of the reagents is based on using the complete 1 X 96 well assay, unless otherwise noted. If only a portion of the immunoassay plate is to be used, please store all components as previously described.*

1. TEMPERATURE OF REAGENTS

Bring the following reagents to room temperature prior to use:

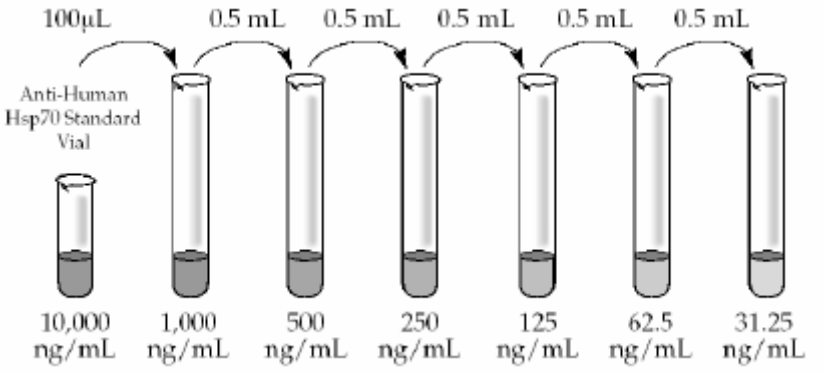
- Rec. Human Hsp70 Immunoassay Plate
- Sample Diluent
- 20X Wash Buffer
- TMB Substrate
- Stop Solution

2. ANTI-HUMAN HSP70 STANDARD

Caution: This standard is derived from human serum. Treat as biohazard.

The Anti-Human Hsp70 Standard is used to generate a standard curve with 6 points, ranging from 31.25 -1000 ng/mL.

a. Centrifuge the Anti-Human Hsp70 Standard vial before removing cap.
b. Label six (6) disposable 12 x 75 mm tubes with the following standard values: 1000 ng/mL, 500 ng/mL, 250 ng/mL, 125 ng/mL, 62.5 ng/mL, 31.25 ng/mL.
c. Add 0.9 mL of Sample Diluent to Tube #1 (1000 ng/mL).
d. Add 0.5 mL of Sample Diluent to Tube #2, 3, 4, 5 and 6.
e. Add 100 µL of the Anti-Human Hsp70 Standard stock (10,000 ng/mL) to Tube #1 (1000 ng/mL).
f. Mix gently.
g. Transfer 0.5 mL from Tube #1 (1000 ng/mL) to Tube #2 (500 ng/mL).
h. Mix gently.
i. Similarly, complete the dilution series to generate the remaining standards (0.5 mL from Tube #2 to Tube #3, mix gently, etc) up to and including Tube #6.



j. Finally, add 0.5 mL Sample Diluent to another 1.5 mL disposable 12 x 75mm tube (Tube #7), which is the assay blank (0 ng/mL).

NOTE: Diluted standards should be used within 60 minutes of preparation.

3. WASH BUFFER

a. Bring the 20X Wash Buffer to room temperature and swirl gently to dissolve any crystals that may have formed from storage.
b. Dilute the 100 mL of 20X Wash Buffer with 1900 mL of deionized or distilled water. Once diluted, the 1X Wash Buffer is stable at room temperature for up to 4 weeks. For longer term storage, the Wash Buffer should be stored at 4°C.

NOTE: *100 mL of 20X Wash Buffer has been provided in this kit, which is sufficient for the preparation of 2L of 1X Wash Buffer. The minimum required volume of 1X Wash Buffer is 310 mL (if the complete plate is used at once). However, additional 1X Wash Buffer is supplied to allow for multiple assays or alternative washing techniques.*

3.4 ASSAY PROCEDURE

1. DETERMINE THE REQUIRED NUMBER OF WELLS
   a. If less than 96 pre-coated microtitre wells are needed, remove the excess wells from the frame and return them to the foil pouch.
   b. Reseal the pouch containing the unused wells and store at 4°C.
2. ADDITION OF STANDARDS, SAMPLES AND BLANK
   a. Add 100 µL (in duplicate) of each of the following to appropriate wells:
      - Prepared anti-human Hsp70 standards (Tube#1 through Tube #6)
      - Samples (previously prepared - see Sample Preparation)
      - 0-Standard (Sample Diluent, which represents 0 ng/mL)
   b. Cover wells with an adhesive plate sealer or plasticwrap and **incubate at room temperature for 2 hours**, preferably with gentle rocking or shaking.
3. WASHING
   a. Aspirate liquid from all wells.
   b. Add 400 µL of 1X Wash Buffer to each well, using a multi-channel pipette, manifold dispenser, automated microplate washer, or a squirt bottle.
   c. Repeat the aspirating and washing 3 more times, for a total of 4 washes.
   d. After the 4th addition of 1X Wash Buffer, aspirate the liquid from all wells. Invert the plate and carefully pat dry on clean paper towels.
4. ADDITION OF ANTI-HUMAN GAM-HRP CONJUGATE
   a. Add 100 µL of the Anti-Human GAM-HRP Conjugate to each well.
   b. Cover wells with a fresh adhesive plate sealer and **incubate at room temperature for 1 hour**, preferably with gentle rocking or shaking.
   c. Wash plate as described in Step #3.
5. ADDITION OF TMB SUBSTRATE AND STOP SOLUTION
   a. Add 100 µL of the TMB Substrate to each well.
   b. Incubate the plate **at room temperature** for approximately **15 minutes**. preferably with gentle rocking or shaking.
   c. Add 100 µL of the Stop Solution to each well in the same order that the TMB Substrate was added.
6. MEASURING ABSORBANCE
   a. Set up the microplate reader according to the manufacturer's instructions.
   b. Set wavelength at 450 nm. If the reader is capable of measuring at dual wavelengths, set the correction wavelength at 540 or 570 nm.
   c. Measure the absorbance. If the plate cannot be read immediately, it should be covered and kept at room temperature. The absorbance should be read within 30 minutes of adding the Stop Solution.

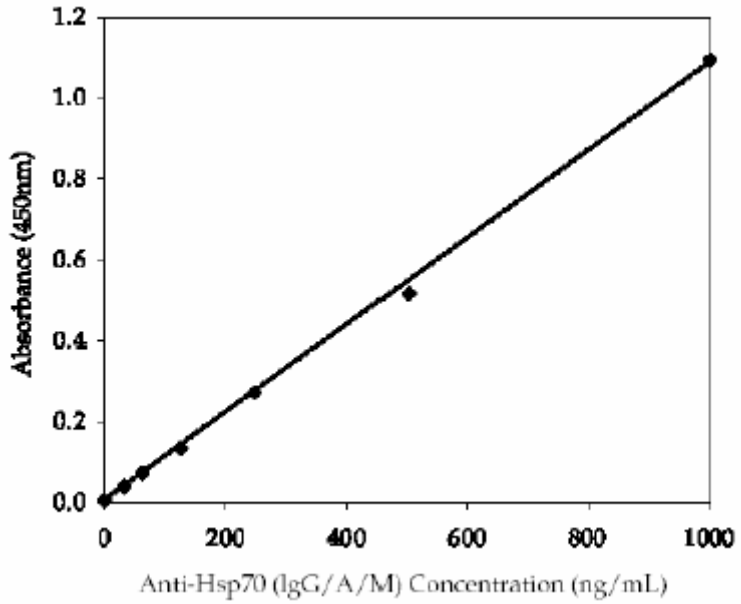
3.5 CALCULATION OF RESULTS -

DETERMINATION OF ANTI-HUMAN HSP70 CONCENTRATIONS

1. Calculate the average of the duplicate absorbance measurements for each standard and sample.
2. Calculate the average of the duplicate absorbance measurements for the 0ng/mLanti-human Hsp70 standard (assay blank).
3. Subtract the average value obtained in Step#2 (0 ng/mL anti-humanHsp70 standard (assay blank)) from the values obtained in Step#1 (standards and samples).
4. To generate the standard curve, plot the anti-human Hsp70 standard concentrations (ng/mL) on the X-axis and the corresponding absorbance measurements on the Y-axis. Determine the best fit line.
5. Interpolate the sample concentrations from the standard curve and multiply by the dilution factor for the final sample anti-human Hsp70 concentration.
   For example, if the sample was diluted 1:25 prior to assaying, the value generated from the standard curve must be multiplied by 25 to calculate the final sample anti-human Hsp70 (IgG/A/M) concentration.

NOTE: *Manufacturers of microplate readers usually offer accompanying software programs that will analyze data, plot standard curves and calculate sample concentrations. To set up the program for calculating the results, consult with the software instruction manual or contact the manufacturer of the microplate reader.*

TYPICAL ANTI-HUMAN HSP70 STANDARD CURVE



4 PERFORMANCE CHARACTERISTICS

4.1 SENSITIVITY

To determine sensitivity of the assay, the mean absorbance value for sixteen replicates of Sample Diluent (0 ng/mL) was compared to the mean absorbance value for sixteen replicates of standard Tube #6 (31.25 ng/mL).

The detection limit was determined as the concentration of anti-human Hsp70 measured at two standard deviations from the 0 ng/mL standard along the standard curve.

The sensitivity of the Anti-Human Hsp70 (lgG/A/M) ELISA was determined to be 6.79 ng/mL.

4.2 PRECISION

a) Intra-Assay Precision (Within Run Precision)

To determine Intra-Assay Precision, samples containing low, medium and high concentrations of anti-human Hsp70 were assayed sixteen times on one plate. The Intra-Assay coefficient of variation of the Anti-Human Hsp70 (lgG/A/M) ELISA was determined to be <10%.

b) Inter-Assay Precision (Between Run Precision)

To determine Inter-Assay Precision, samples containing low, medium and high concentrations of anti-human Hsp70 were assayed in eight individual assays. The Inter-Assay coefficient of variation of the Anti-Human Hsp70 (lgG/A/M) ELISA was determined to be <10%.

4.3 LINEARITY

To determine linearity, a sample containing 792.0 ng/mL of anti-human Hsp70 was diluted 1:2 in Sample Diluent four times and measured in the assay. The data was plotted graphically as actual anti-human Hsp70 concentration versus measured anti-human Hsp70 concentration.

The line obtained had a slope of 1.071 and a correlation coefficient of 0.9998.

4.4 LIMITATIONS OF THE ASSAY

- This assay has been validated for use with serum. Other sample types or matrices (e.g. tissue extracts, cell lysates, urine, cerebrospinal fluid, cell culture supernatant, etc.) may contain interfering factors that can compromise the performance of the assay, or produce inaccurate results.
- If serum samples generate greater values than the highest standard, the samples should be re-assayed at a higher sample dilution. Similarly, if samples generate lower values than the lowest standard, the samples should be re-assayed at a lower sample dilution.
- The use of assay reagents not provided in this kit or amendments to the protocol can compromise the performance of this assay.
- The components in each kit lot number have been quality assured and warranted in this specific combination only; please do not mix them with components from other kit lot numbers.

5 REFERENCES

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6 APPENDIX

6.1 Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	125 ng/mL										
B	Blank	125 ng/mL										
C	1000 ng/mL	62.5 ng/mL										
D	1000 ng/mL	62.5 ng/mL										
E	500 ng/mL	31.25 ng/mL										
F	500 ng/mL	31.25 ng/mL										
G	250 ng/mL											
H	250 ng/mL											

SYMBOLS USED WITH DRG ASSAYS

Symbol	English	Deutsch	Français	Español	Italiano
	Consult instructions for use	Gebrauchsanweisung beachten	Consulter les instructions d'utilisation	Consulte las instrucciones de uso	Consultare le istruzioni per l'uso
CE	European Conformity	CE-Konfirmitäts-kennzeichnung	Conformité aux normes européennes	Conformidad europea	Conformità europea
IVD	In vitro diagnostic device	In-vitro-Diagnostikum	Usage Diagnostic in vitro	Para uso Diagnóstico in vitro	Per uso Diagnostica in vitro
RUO	For research use only	Nur für Forschungszwecke	Seulement dans le cadre de recherches	Sólo para uso en investigación	Solo a scopo di ricerca
REF	Catalogue number	Katalog-Nr.	Numéro de catalogue	Número de catálogo	Numero di Catalogo
LOT	Lot. No. / Batch code	Chargen-Nr.	Numéro de lot	Número de lote	Numero di lotto
	Contains sufficient for <n> tests/	Ausreichend für "n" Ansätze	Contenu suffisant pour "n" tests	Contenido suficiente para <n> ensayos	Contenuto sufficiente per "n" saggi
	Storage Temperature	Lagerungstemperatur	Température de conservation	Temperatura de conservación	Temperatura di conservazione
	Expiration Date	Mindesthaltbarkeits-datum	Date limite d'utilisation	Fecha de caducidad	Data di scadenza
	Legal Manufacturer	Hersteller	Fabricant	Fabricante	Fabbricante
Distributed by	Distributor	Vertreiber	Distributeur	Distribuidor	Distributore
Content	Content	Inhalt	Conditionnement	Contenido	Contenuto
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantité	Volumen/Número	Volume/Quantità

Symbol	Portugues	Dansk	Svenska	Ελληνικά
	Consulte as instruções de utilização	Se brugsanvisning	Se bruksanvisningen	Εγχειρίδιο χρήστη
CE	Conformidade com as normas europeias	Europaeisk overensstemmelse	Europeisk överensstämmelse	Ευρωπαϊκή Συμμόρφωση
IVD	Diagnóstico in vitro	In vitro diagnostik	Diagnostik in vitro	in vitro διαγνωστικό
RUO				
REF	Catálogo n.º	Katalognummer	Katalog nummer	Αριθμός καταλόγου
LOT	No do lote	Lot nummer	Batch-nummer	Αριθμός Παρτίδος
		Indeholder tilsttrækkeligt til "n" test	Innehåller tillräckligt till "n" tester	Περιεχόμενο επαρκές για «n» εξετάσεις
	Temperatura de conservação	Opbevarings-temperatur	Förvaringstempratur	Θερμοκρασία αποθήκευσης
	Prazo de validade	Udløbsdato	Bäst före datum	Ημερομηνία λήξης
	Fabricante	Producent	Tillverkare	Κατασκευαστής
Distributed by				
Content	Conteúdo	Indhold	Innehåll	Περιεχόμενο
Volume/No.	Volume/Número	Volumen/antal	Volym/antal	Όγκος/αριθ..