

DRG[®] Human p27 ELISA (EIA-4491)

Revised 12 May 2009 (Vers. 2.0)

RUO**FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.****1 INTRODUCTION**

The p27 (Human) ELISA kit is a complete kit for the quantitative determination of p27^{Kip1} in cell lysates. Please read the complete kit insert before performing this assay.

p27 is also known as, KIP1, p27^{Kip1}, cyclin-dependent kinase inhibitor 1B (CDKN1B), and cyclin-dependent kinase inhibitor p27.

It is composed of 198 amino acids and has a calculated molecular weight of 22 kDa. p27^{Kip1} is a member of a family of CDK inhibitors (CDIs) that binds to cyclin/CDK complexes and arrests cell division. In general, p27^{Kip1} expression is highest in quiescent cells and falls as cells reenter the cell cycle. The p27^{Kip1} protein was first identified as an inhibitor of cyclin E-CDK2 [3]. Several anti-proliferative signals, including cell-to-cell contact, the withdrawal of cytokines, the withdrawal of mitogens, and exposure to cAMP [4], result in p27^{Kip1} accumulation. p27^{Kip1} has been implicated in several fundamental cellular processes including apoptosis, cell division, and proliferation. The p27^{Kip1} gene is a tumor-suppressor that plays a crucial role in the pathogenesis of human malignancy [5]. A decrease in p27^{Kip1} expression has been associated with aggressive growth of breast, lung, colorectal, gastric, and prostate tumors [6-10].

2 PRINCIPLE

1. Samples and standards are added to wells coated with a monoclonal antibody specific for p27Kip1. The plate is then incubated.
2. The plate is washed, leaving only bound p27Kip1 on the plate. A yellow solution of polyclonal antibody to human p27Kip1 is then added. This binds the p27Kip1 captured on the plate. The plate is then incubated.
3. The plate is washed to remove excess antibody. A blue solution of HRP conjugate is added to each well, binding to the p27Kip1 polyclonal. The plate is again incubated.
4. The plate is washed to remove excess HRP conjugate. TMB Substrate solution is added. The substrate generates a blue color when catalyzed by the HRP.
5. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is directly proportional to the level of p27Kip1 in the sample

3 MATERIALS SUPPLIED

Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.

1. RIPA Cell **Lysis Buffer**, 2 bottles, each containing 105 mL,
50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% Sodium Deoxycholate and 0.1% SDS
2. Human p27^{Kip1} **Standard**, 0.150 mL,
A solution of 64,000 pg/mL recombinant p27^{Kip1}.
The standard should be handled with care due to the known and unknown effects of the molecule.

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3. .p27^{Kip1} Clear **Microtiter Plate**, One Plate of 96 Wells,
A plate of break-apart strips coated with a mouse monoclonal antibody raised against amino acids 1-197 of mouse Kip1.
4. **Wash Buffer Concentrate**, 20x, 105 mL
Tris buffered saline containing detergents.
5. Total p27^{Kip1} EIA **Antibody**, 11 mL
A yellow solution of rabbit polyclonal antibody raised against a peptide mapping to the carboxy terminus of human p27.
6. Total p27^{Kip1} EIA **Conjugate**, 11 mL,
A blue solution of goat anti-rabbit IgG conjugated to Horseradish peroxidase.
Activity of conjugate is affected by nucleophiles such as azide, cyanide and hydroxylamine.
7. **TMB Substrate**, 11 mL,
A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide.
Protect substrate from prolonged exposure to light.
8. **Stop Solution**, 11 mL,
A 1N solution of hydrochloric acid in water.
Stop solution is caustic. Keep tightly capped.
9. **Assay Layout Sheet**, 1 each,
10. **Plate Sealer**, 3 each,

4 STORAGE

All components of this kit, **except the Standard**, are stable at 4 °C until the kit's expiration date.

The Standard must be stored at or below –20 °C.

5 MATERIALS NEEDED BUT NOT SUPPLIED

1. Deionized or distilled water.
2. Protease Inhibitor Cocktail (PIC), Sigma # P8340 or equivalent.
3. Precision pipets for volumes between 50 µL and 1,000 µL.
4. Repeater pipet for dispensing 100 µL.
5. Disposable beakers for diluting buffer concentrates.
6. Graduated cylinders.
7. A microplate shaker.
8. Lint-free paper for blotting.
9. Microplate reader capable of reading at 450 nm.
10. Graph paper for plotting the standard curve.

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6 REAGENT PREPARATION

Bring all reagents, except RIPA Cell Lysis Buffer, to room temperature for at least 30 minutes prior to opening. RIPA Cell Lysis Buffer is best used cold.

Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

1. Wash Buffer

Prepare the Wash Buffer by diluting 50 mL of the supplied concentrate with 950 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. PIC Addition

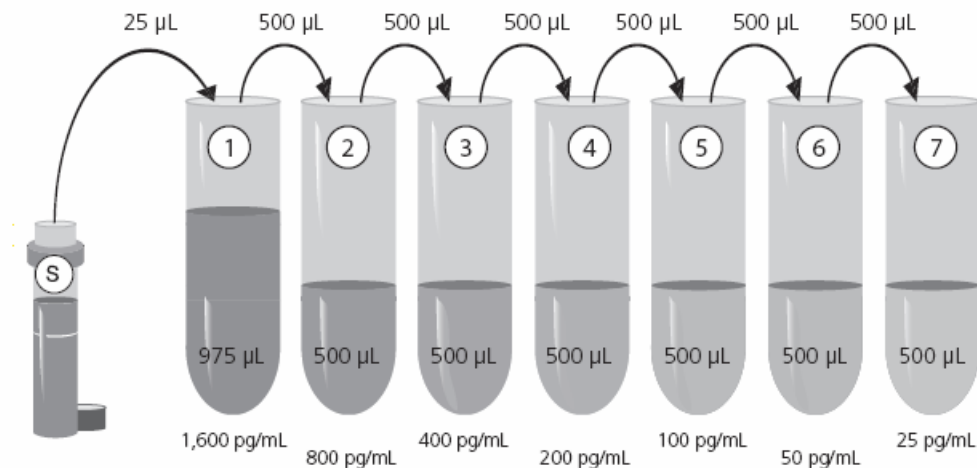
Immediately prior to use, PIC must be added to the RIPA Cell Lysis buffer. If using Sigma Protease Inhibitor Cocktail (PIC) #P8340, add 0.5 µL/mL PIC or equivalent concentration according to alternate vendor’s spec sheet.

Inhibitors must be freshly added to the RIPA Cell Lysis Buffer to ensure optimal integrity of the standards and samples. Each inhibitor treated buffer should incubate for 5-10 minutes at room temperature before it is used. Buffers treated with inhibitors should be used within 1 hour of preparation

If inhibitors other than those recommended are used, the end-user is responsible for assay validation. In some cases, certain protease inhibitor cocktails may cause performance differences.

3. human p27^{Kip1} Standards

Plastic tubes must be used for standard preparation.



Label seven 12 x 75 mm polypropylene tubes #1 through #7.

Pipet 975 µL of Assay Buffer into tube #1.

Pipet 500 µL of Assay Buffer into tubes #2 through #7.

Add 25 µL of the 64,000 pg/mL standard stock into tube #1 and vortex thoroughly.

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Add 500 µL of tube #1 to tube #2 and vortex thoroughly..

Add 500 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #7.

Diluted standards should be used within 30 minutes of preparation.

Standards and samples should be prepared on ice, with the human p27^{Kip1} Standard thawed for the minimum time possible.

The concentrations of p27^{Kip1} in tubes are labeled above.

7 SAMPLE HANDLING

If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.

Samples must be stored frozen at or below -20°C to avoid loss of bioactive analyte.

Repeated freeze/thaw cycles should be avoided.

This assay is suitable for measuring p27^{Kip1} in a wide range of cell lysates. Prior to assay, frozen samples should be slowly brought to 4°C and centrifuged, if necessary, to isolate residual cell debris.

Due to differences in cell types, number of cells, or total cellular protein concentration, lysates may require dilution with RIPA Cell Lysis Buffer plus inhibitors to remove interference or to be read within the standard range. Below are examples of the lysis of human HeLa cells. In general, ≤ 20 µg total cellular protein is a good starting concentration.

Sample	# cells per mL of lysis buffer	Total cellular protein (mg/mL)	% Recovery	Recommended Dilution
HeLa cells	1.74 million	4.27	100.9%	1:160
HeLa cells	1.07 million	2.72	96.6%	1:80

8 PROTOCOL FOR CELL LYSIS

Add PIC to buffers prior to preparing samples.

1. Harvest cells and centrifuge at 7,000 rpm for 10 minutes at 4°C. Discard supernatant.
2. Resuspend pellet and wash with Hank's Balanced Salt Solution.
3. Centrifuge at 7,000 rpm for 10 minutes at 4°C. Discard supernatant.
4. Resuspend pellet with RIPA Cell Lysis Buffer 3 plus inhibitors. Vortex and incubate on ice for 30 minutes.
5. Centrifuge at 16,000 x g for 20 minutes at 4°C. The supernatants can be stored at or below -20°C or used immediately in the assay.

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9 ASSAY PROCEDURE

Bring all reagents, except RIPA Cell Lysis Buffer, to room temperature for at least 30 minutes prior to opening. RIPA Cell Lysis Buffer is best used cold.

All standards, controls, and samples should be run in duplicate.

Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

Pipet the reagents to the sides of the wells to avoid possible contamination.

Prior to the addition of antibody, conjugate, and substrate, ensure there is no residual wash buffer in the wells.

Remaining wash buffer may cause variation in assay results

Refer to the Assay Layout Sheet to determine the number of wells to be used.

Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal.

Store unused wells at 4 °C.

1. Pipet 100 µL of assay buffer into the S0 (0 pg/mL standard) wells.
2. Pipet 100 µL of standards #1 through #7 to the bottoms of the appropriate wells.
3. Pipet 100 µL of the samples to the bottom of the appropriate wells.
4. Seal the plate. Incubate for 2 hours on a plate shaker (~500 rpm) at room temperature
5. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
6. Pipet 100 µL of yellow Antibody into each well, except the Blank.
7. Seal the plate. Incubate for 1 hour on a plate shaker (~500 rpm) at room temperature.
8. Wash as above (Step 5).
9. Add 100 µL of blue Conjugate to each well, except the Blank.
10. Seal the plate. Incubate for 30 minutes on a plate shaker (~500 rpm) at room temperature.
11. Wash as above (Step 5).
12. Pipet 100 µL of Substrate Solution into each well.
13. Incubate for 30 minutes on a plate shaker at (~500 rpm) at room temperature
14. Pipet 100 µL Stop Solution to each well.
15. After blanking the plate reader against the substrate blank, read optical density at 450 nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

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10 CALCULATION OF RESULTS

Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

Several options are available for the calculation of the concentration of p27^{Kip1} in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentrations can be calculated as follows:

1. Calculate the average net Optical Density (OD) for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

$$\text{Average Net OD} = \text{Average OD} - \text{Average Blank OD}$$

2. Using linear graph paper, plot the Average Net OD for each standard versus p27^{Kip1} concentration in each standard. Approximate a straight line through the points. The concentration of p27^{Kip1} in the unknowns can be determined by interpolation.

11 TYPICAL RESULTS

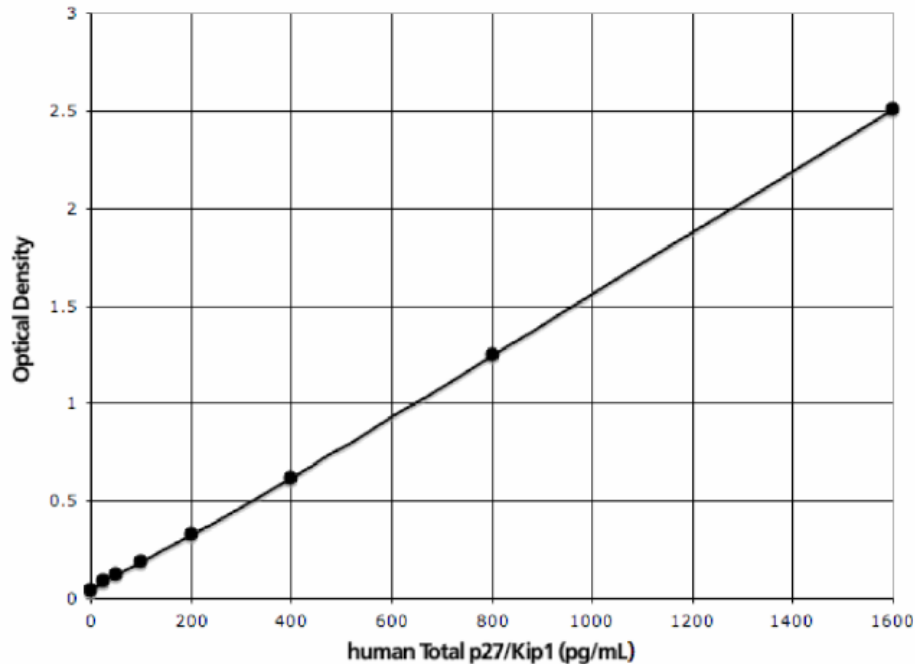
The results shown below are for illustration only and should not be used to calculate results from another assay.

Sample	Net OD	p27 ^{Kip1} (pg/mL)
S0	0.046	0
S1	2.504	1,600
S2	1.249	800
S3	0.618	400
S4	0.325	200
S5	0.187	100
S6	0.122	50
S7	0.088	25
Unknown 1	0.884	569
Unknown 2	0.250	147

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12 PERFORMANCE CHARACTERISTICS

12.1 Specificity

The cross reactivities for a number of related compounds were determined by dissolving cross reactants in the assay buffer at a concentration of 160,000 pg/mL. These samples were then measured in the assay.

Compound	Cross Reactivity
human p27 ^{Kip1}	100%
Aurora A	< 0.15%
Bcl-2	< 0.15%
PCNA	< 0.15%
p16 Ink4a	< 0.15%
p21	< 0.15%

12.2 p27^{Kip1} Down Regulation Experiment

The number of HeLa cells lysed in this experiment was 10.75 million per mL for the non-target control cells and 6.69 million per mL for the siRNA treated cells.

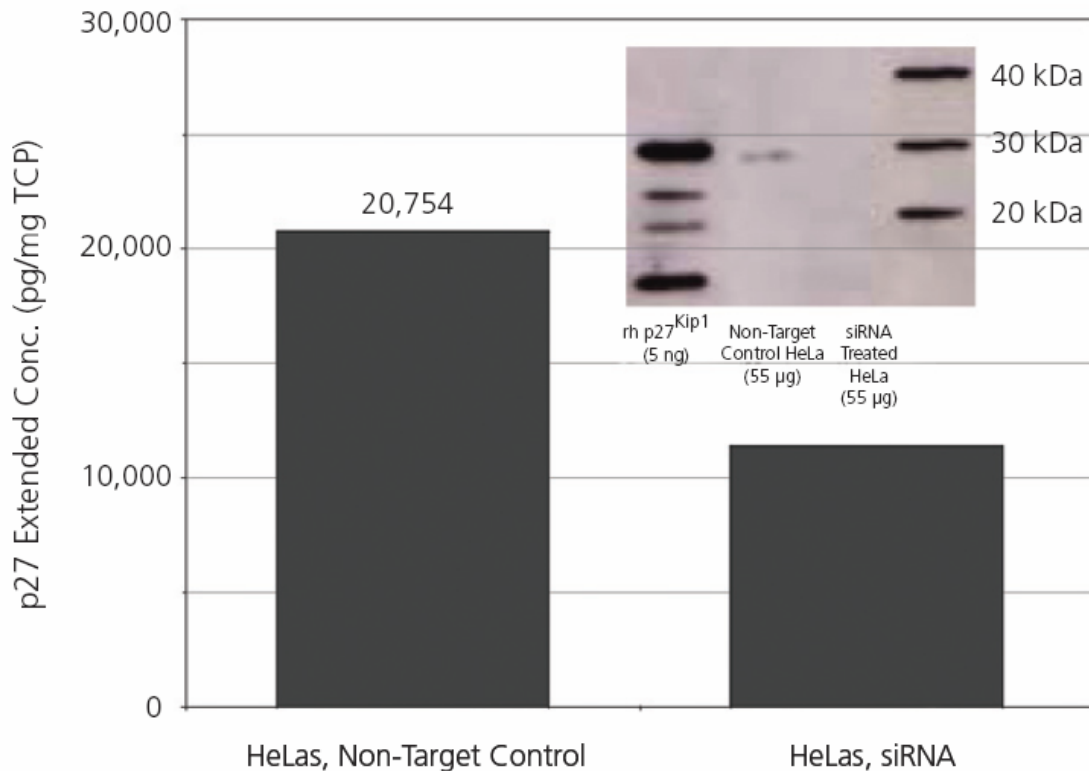
The total protein concentrations for the control group and siRNA treated group were 4.27 mg/mL and 2.72 mg/mL, respectively.

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The treated HeLa cells were transfected with p27-targeting siRNA and the control HeLa cells were transfected with a non-targeting siRNA. The transfected cells were incubated at 37 °C for 3 days. On the fourth day, cells were lysed according to the protocol on page 6. Lysates were run in the Western blot and EIA to generate the data illustrated. In the Western blot, 55 µg of cell Lysate were landed in each lane. 5 ng of recombinant p27^{Kip1} were run as a positive control. The kit capture antibody was used as the detection antibody for the Western blot.



12.3 Sensitivity

The sensitivity of the assay, defined as the concentration of p27^{Kip1} measured at 2 standard deviations from the mean of 16 zeros along the standard curve, was determined to be 10.1 pg/mL.

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12.4 Linearity

A buffer sample containing p27^{Kip1} was serially diluted 1:2 in the assay buffer and measured in the assay. The results are shown in the table below.

Dilution	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
Neat	---	1,286 pg/mL	---
1:2	642.8 pg/mL	644.1 pg/mL	100.2 %
1:4	321.4 pg/mL	325.2 pg/mL	101.2 %
1:8	160.7 pg/mL	155.2 pg/mL	96.6 %
1:16	80.3 pg/mL	82.2 pg/mL	102.4 %
1:32	40.2 pg/mL	37.4 pg/mL	93.0 %

12.5 Precision

Intra-assay precision was determined by assaying 24 replicates of three buffer controls containing p27Kip1 in a single assay.

pg/mL	%CV
146	5.4
548	2.7
1,074	1.6

Inter-assay precision was determined by measuring buffer controls of varying p27Kip1 concentrations in multiple assays over several days.

pg/mL	%CV
147	6.7
569	2.6
1,078	10.4

13 REFERENCES

1. K. Polyak, et al. Genes Dev. (1994) 8: 9-22.
2. J.Y. Kato, et al. Cell. (1994) 79: 487-96.
3. A. Sgambato, et al. J. Cell. Physiol. (2000) 183: 18-27.
4. K. Keyomarsi, et al. Cancer Res. (1994) 54: 380-5.
5. H. Kawana, et al. Am. J. Pathol. (1998) 153: 505-13.
6. M. Loda, et al. Nat. Med. (1997) 3: 231-4.



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7. M. Ohtani, et al. Cancer. (1999) 85: 1711-8.
8. J. Tsihlias, et al. Cancer Res. (1998) 58: 542-8.

14 LIMITED WARRANTY

DRG warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.





















DRG must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if DRG is not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

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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
	European Conformity	CE-Konformitätskennzeichnung	Conformité aux normes européennes	Conformidad europea	Conformità europea
	In vitro diagnostic device	In-vitro-Diagnostikum	Usage Diagnostic in vitro	Para uso Diagnóstico in vitro	Per uso Diagnostica in vitro
	For research use only	Nur für Forschungszwecke	Seulement dans le cadre de recherches	Sólo para uso en investigación	Solo a scopo di ricerca
	Catalogue number	Katalog-Nr.	Numéro de catalogue	Número de catálogo	Numero di Catalogo
	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	
	Conformidade com as normas europeias	Europaeisk overensstemmelse	Europeisk överensstämmelse	Ευρωπαϊκή Συμμόρφωση	
	Diagnóstico in vitro	In vitro diagnostik	Diagnostik in vitro	in vitro διαγνωστικό	
					
	Catálogo n.º	Katalognummer	Katalog nummer	Αριθμός καταλόγου	
	No do lote	Lot nummer	Batch-nummer	Αριθμός Παρτίδος	
		Indeholder tilstrækkeligt til "n" test	Innehåller tillräckligt till "n" tester	Περιεχόμενο επαρκές για «n» εξετάσεις	
	Temperatura de conservação	Opbevarings-temperatur	Förvaringstemperatur	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. .	