ELISA Kit
Catalog #KHO0201

**AKT*
[pT308]**

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*Patent Pending

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INTRODUCTION

AKT, also known as the protein kinase B-α (PKB-α) or RAC-PKα, was initially identified as one of the downstream targets of PI-3 Kinase (PI3-K). AKT is now known to consist of three highly conserved isoforms, which are designated in humans as AKT1, AKT2, and AKT3. Each isoform consists of an N-terminus pleckstrin homology (PH) domain, which mediates lipid-protein or protein-protein interactions, and a C-terminus kinase catalytic domain. Although each kinase is expressed differentially in a tissue-specific manner, they respond in a similar fashion to various stimuli.

AKT can be activated by a diverse array of growth factors and physiologic stimuli in a PI3-K-dependent manner. Activation of AKT kinase is a multi-step process involving both membrane translocation and phosphorylation. Activated PI3-K generates 3' phosphoinositide products, 3,4,5-triphosphates (PI-3,4,5-P_3) and PI-3,4-P_2. AKT is recruited from the cytosol to the plasma membrane through the interaction of its PH domain with these phosphoinositides. Upon membrane localization, AKT undergoes a conformational change, which makes it accessible to phosphorylation at threonine-308 and serine-473 in the kinase domain by PDK-1 and related kinases. Activated AKT then acts as a key mediator of signals for cell survival, proliferation, angiogenesis, and a number of metabolic effects of insulin. The effects of AKT activation may be mediated by modulation of expression or activity of various molecules including Bcl-2, BAD, caspase-9, endothelial nitric oxide synthase (eNOS), glycogen synthase, and transcription factors (NF-kB, Forkhead, CREB, Mdm2).

The Invitrogen AKT [pT308] ELISA is designed to detect and quantify the level of AKT protein that is phosphorylated at threonine residue

308. This assay is intended for the detection of AKT [pT308] from lysates of cells. For normalizing the AKT content of the samples, an AKT (Total) ELISA kit, which is independent of phosphorylation status, is available from Invitrogen (Cat. # KHO0101).

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READ ENTIRE PROTOCOL BEFORE USE

PRINCIPLE OF THE METHOD

The Invitrogen AKT [pT308] kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for AKT (regardless of phosphorylation state) has been coated onto the wells of the microtiter strips provided. Samples, including a standard containing AKT [pT308], control specimens, and unknowns, are pipetted into these wells. During the first incubation, the AKT antigen binds to the immobilized (capture) antibody. After washing, a rabbit antibody, specific for AKT phosphorylated at threonine 308, is added to the wells. During the second incubation, this antibody serves as a detector by binding to the immobilized AKT protein captured during the first incubation. After removal of excess detection antibody, a horseradish peroxidase-labeled Anti-Rabbit IgG (Anti-Rabbit IgG HRP) is added. This binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove all the excess Anti-Rabbit IgG HRP, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of AKT [pT308] present in the original specimen.

REAGENTS PROVIDED

Note: *Store all reagents at 2 to 8°C.*

Reagent	***96 Test Kit***
AKT [pT308] Standard: Lyophilized. Contains 0.1% sodium azide. Refer to vial label for quantity and reconstitution volume.	4 vials
Standard Diluent Buffer. Contains 0.1% sodium azide; 25 mL per bottle.	1 bottle
*AKT Antibody Coated Well*s. 96 Well Plate.	1 plate
AKT [pT308] Detection Antibody. Contains 0.1% sodium azide; 11 mL per bottle.	1 bottle
Anti-Rabbit IgG HRP (100X). Contains 3.3 mM thymol; 0.125 mL per vial.	1 vial
HRP Diluent. Contains 3.3 mM thymol; 25 mL per bottle.	1 bottle
Wash Buffer Concentrate (25X). 100 mL per bottle.	1 bottle
*Stabilized Chromoge*n, *Tetramethylbenzidine (TMB).* 25 mL per bottle.	1 bottle
*Stop Solutio*n. 25 mL per bottle.	1 bottle
Plate Covers, adhesive strips.	3

Disposal Note: This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

SUPPLIES REQUIRED BUT NOT PROVIDED

1. Microtiter plate reader capable of measurement at or near 450 nm.
2. Calibrated adjustable precision pipettes, preferably with disposable plastic tips. (A manifold multi-channel pipette is desirable for large assays.)
3. Cell Extraction Buffer (see Recommended Formulation, p. 11).
4. Distilled or deionized water.
5. Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
6. Data analysis and graphing software. Graph paper: linear (Cartesian), log-log, or semi-log, as desired.
7. Glass or plastic tubes for diluting and aliquoting standard.
8. Absorbent paper towels.
9. Calibrated beakers and graduated cylinders in various sizes.

PROCEDURAL NOTES/LAB QUALITY CONTROL

1. When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
2. **Microtiter plates should be allowed to come to room temperature before opening the foil bags.** Once the desired number of strips has been removed, immediately reseal the bag and store at 2 to 8°C to maintain plate integrity.

3. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
4. If particulate matter is present, centrifuge or filter prior to analysis.
5. All standards, controls and samples should be run in duplicate.
6. Samples containing *AKT [pT308]* protein extracted from cells should be diluted with *Standard Diluent Buffer* at least 1:10. This dilution is necessary to reduce the matrix effect of the cell lysis buffer. SDS concentration should be less than 0.01% before adding to the plate.
7. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
8. Cover or cap all reagents when not in use.
9. **Do not mix or interchange different reagent lots from various kit lots**.
10. Do not use reagents after the kit expiration date.
11. Read absorbances within 2 hours of assay completion.
12. In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
13. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. ***Never*** insert absorbent paper directly into the wells.
14. Because *Stabilized Chromogen* is light sensitive, avoid prolonged exposure to light. Also avoid contact between *Stabilized Chromogen* and metal, or color may develop.

SAFETY

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

DIRECTIONS FOR WASHING

Incomplete washing will adversely affect the test outcome. All washing must be performed with *Wash Buffer Concentrate (25X)* provided.

Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into the bottom of each well. Take care not to scratch the inside of the well.

After aspiration, fill the wells with at least 0.4 mL of diluted wash solution. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under **ASSAY METHOD**. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

Alternatively, the wash solution may be put into a squirt bottle. If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

If using an automated washer, the operating instructions for washing equipment should be carefully followed. If your automated washer allows, 30 second soak cycles should be programmed into the wash cycle.

PROCEDURE FOR EXTRACTION OF PROTEINS FROM CELLS

A. Recommended Formulation of Cell Extraction Buffer:

10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM $Na_4P_2O_7$
2 mM Na_3VO_4
1% Triton X-100
10% glycerol
0.1% SDS
0.5% deoxycholate
1 mM PMSF (stock is 0.3 M in DMSO)
Protease inhibitor cocktail (e.g., Sigma Cat. # P-2714) (reconstituted according to manufacturer's guideline). Add 250 µL per 5 mL Cell Extraction Buffer.

This buffer is stable for 2 to 3 weeks at 4°C or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at -20°C. When stored frozen, the Cell Extraction Buffer should be thawed on ice. Important: add the protease inhibitors just before using. The stability of protease inhibitor supplemented Cell Extraction Buffer is 24 hours at 4°C. PMSF is very unstable and must be added prior to use, even if added previously.

This buffer (minus protease inhibitor) is available from Invitrogen (Cat. # FNN0011).

B. Protocol for Cell Extraction

This protocol has been successfully applied to several cell lines of human, mouse or rat origin. Researchers should optimize the cell extraction procedures for their own applications.

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash cells twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at -80°C and lysed at a later date.)
4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes, on ice, with vortexing at 10 minute intervals. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of AKT [pT308]. For example, 4 x 10^7 Jurkat cells grown in RPMI plus 10% FBS can be extracted in 1 mL of Extraction Buffer. Under these conditions, use of 0.1-5 µL of the clarified cell extract diluted to a volume of 100 µL/well in Standard Diluent Buffer (See **Assay Method**) is sufficient for the detection of AKT [pT308].
5. Transfer extract to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4°C.
6. Aliquot the clear lysate to clean microfuge tubes. These samples are ready for assay. Lysates can be stored at -80°C. Avoid multiple freeze/thaw cycles.

REAGENT PREPARATION AND STORAGE

A. Reconstitution and Dilution of AKT [pT308] Standard

Note: This *AKT [pT308] Standard* is prepared using purified, full length, human recombinant AKT expressed in *Sf*21 cells. One Unit of standard is defined as the amount of AKT [pT308] derived from 500 pg of AKT, which was phosphorylated by MAPKAP2 and PDK1. Subsequent lots of standard will be normalized to this lot of material to allow consistency of AKT [pT308] quantitation.

1. Reconstitute *AKT [pT308] Standard* with *Standard Diluent Buffer*. Refer to standard vial label for instructions. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Label as 100 Units/mL AKT [pT308]. Use standard within 1 hour of reconstitution.
2. Add 0.25 mL of *Standard Diluent Buffer* to each of 6 tubes labeled 50, 25, 12.5, 6.25, 3.12 and 1.6 Units/mL AKT [pT308].
3. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

B. Dilution of AKT [pT308] Standard

Standard:	Add:	Into:
100 Units/mL	Prepare as described in step 1	
50 Units/mL	0.25 mL of the 100 Units/mL std.	0.25 mL of the Diluent Buffer
25 Units/mL	0.25 mL of the 50 Units/mL std.	0.25 mL of the Diluent Buffer
12.5 Units/mL	0.25 mL of the 25 Units/mL std.	0.25 mL of the Diluent Buffer
6.25 Units/mL	0.25 mL of the 12.5 Units/mL std.	0.25 mL of the Diluent Buffer
3.12 Units/mL	0.25 mL of the 6.25 Units/mL std.	0.25 mL of the Diluent Buffer
1.6 Units/mL	0.25 mL of the 3.12 Units/mL std.	0.25 mL of the Diluent Buffer
0 Units/mL	0.25 mL of the Diluent Buffer	An empty tube

Remaining reconstituted standard should be discarded or frozen at -80^{o}C. for further use. Standard can be frozen and thawed one time only without loss of immunoreactivity.

C. Storage and Final Dilution of Anti-Rabbit IgG HRP (100X)

Please Note: The *Anti-Rabbit IgG HRP (100X)* is in 50% glycerol. This solution is viscous. To ensure accurate dilution, allow *Anti-Rabbit IgG HRP (100X)* to reach room temperature. Gently mix. Pipette *Anti-Rabbit IgG HRP (100X)* slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper.

1. Within 1 hour of use, dilute 10 µL of this 100X concentrated solution with 1 mL of *HRP Diluent* for each 8-well strip used in the assay. Label as Anti-Rabbit IgG HRP Working Solution.

For Example:

# of 8-Well Strips	Volume of Anti-Rabbit IgG HRP (100X)	Volume of Diluent
2	20 µL solution	2 mL
4	40 µL solution	4 mL
6	60 µL solution	6 mL
8	80 µL solution	8 mL
10	100 µL solution	10 mL
12	120 µL solution	12 mL

2. Return the unused *Anti-Rabbit IgG HRP (100X)* to the refrigerator.

D. Dilution of Wash Buffer

Allow the *Wash Buffer Concentrate (25X)* to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the *Wash Buffer Concentrate (25X)* with 24 volumes of deionized water (e.g., 50 mL may be diluted up to 1.25 liters, 100 mL may be diluted up to 2.5 liters). Label as Working Wash Buffer.

Store both the concentrate and the Working Wash Buffer in the refrigerator. The diluted buffer should be used within 14 days.

ASSAY METHOD: PROCEDURE AND CALCULATIONS

Be sure to read the *Procedural Notes/Lab Quality Control* section before carrying out the assay.

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

Note: A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)
2. Add 100 µL of the *Standard Diluent Buffer* to zero wells. Well(s) reserved for chromogen blank should be left empty.
3. Add 100 µL of standards, samples or controls to the appropriate microtiter wells. Samples prepared in Cell Extraction Buffer must be diluted 1:10 or greater in *Standard Diluent Buffer* (for example, 10 µL sample into 90 µL buffer). While a 1:10 sample dilution has been found to be satisfactory, higher dilutions such as 1:25 or 1:50 may be optimal. The dilution chosen should be optimized for each

experimental system. Tap gently on side of plate to thoroughly mix. (See **REAGENT PREPARATION AND STORAGE**, Section B.)

4. Cover wells with *plate cover* and incubate for **2 hours at room temperature**. Alternatively, the plate may be incubated overnight at 4°C.
5. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
6. Pipette 100 µL of *AKT [pT308] Detection Antibody* solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix.
7. Cover wells with *plate cover* and incubate for **1 hour at room temperature.**
8. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
9. Add 100 µL *Anti-Rabbit IgG HRP* Working Solution to each well except the chromogen blank(s). (Prepare the working dilution as described in **REAGENT PREPARATION AND STORAGE**, Section C.)
10. Cover wells with the *plate cover* and incubate for **30 minutes at room temperature**.
11. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
12. Add 100 µL of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.

13. Incubate for **30 minutes at room temperature and in the dark**. ***Please Note*: Do not cover the plate with aluminum foil or metalized mylar**. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits of the instrument. The O.D. values at 450 nm can only be read after the *Stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.
14. Add 100 µL of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
15. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µL each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 2 hours after adding the *Stop Solution.*
16. Plot on graph paper the absorbance of the standards against the standard concentration. (Optimally, the background absorbance may be subtracted from *all* data points, including standards, unknowns and controls, prior to plotting.) Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provides the best curve fit.
17. Read the AKT [pT308] concentrations for unknown samples and controls from the standard curve plotted in step 16. **Multiply value(s) obtained for sample(s) by dilution factor to correct for the dilution in step 3.** (Samples producing signals higher than the

highest standard (100 Units/mL) should be further diluted in *Standard Diluent Buffer* and reanalyzed, multiplying the concentration by the appropriate dilution factor.)

18. Values of AKT [pT308] should be normalized for Total AKT content by parallel measurement with the Invitrogen AKT (Total) ELISA Kit (Cat. # KHO0101).

TYPICAL DATA

The following data were obtained for the various standards over the range of 0 to 100 Units/mL AKT [pT308].

AKT [pT308] Standard (Units/mL)	Optical Density (450 nm)
0	0.181
1.6	0.214
3.12	0.259
6.25	0.339
12.5	0.511
25	0.874
50	1.388
100	2.513

LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the 100 Units/mL standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute samples >100 Units/mL with *Standard Diluent Buffer;* reanalyze these and multiply results by the appropriate dilution factor.

The influence of various extraction buffers has not been thoroughly investigated. The rate of degradation of native AKT or dephosphorylation of AKT [pT308] in various matrices has not been investigated. Although AKT degradation or dephosphorylation of AKT [pT308] in the Cell Extraction Buffer described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

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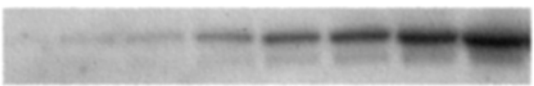
PERFORMANCE CHARACTERISTICS

SENSITIVITY

The analytical sensitivity of this assay is <1.6 Units/mL of AKT [pT308]. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times. The sensitivity of this ELISA was compared to Western blotting using known quantities of AKT [pT308]. The data presented in Figure 1 show that the sensitivity of the ELISA is approximately the same as that of Western blotting. The bands shown in the Western blotting data were developed using rabbit anti-AKT [pT308] (Invitrogen Cat. # 44-602G), an alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography.

Figure 1: Detection of AKT [pT308] by ELISA vs Western Blot:



Western Blot (58 kDa)								
ELISA (O.D. 450 nm)	0.158	0.188	0.216	0.304	0.469	0.827	1.357	2.453
AKT [pT308] (Units/test)	0	0.156	0.312	0.625	1.25	2.5	5	10

PRECISION

1. Intra-Assay Precision

Samples of known AKT [pT308] concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (Units/mL)	42.6	9.0	2.0
SD	1.7	0.3	0.1
%CV	4.1	3.0	3.0

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 36 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (Units/mL)	39	9.0	2.0
SD	2.1	0.5	0.1
%CV	5.4	5.2	5.9

SD = Standard Deviation
CV = Coefficient of Variation

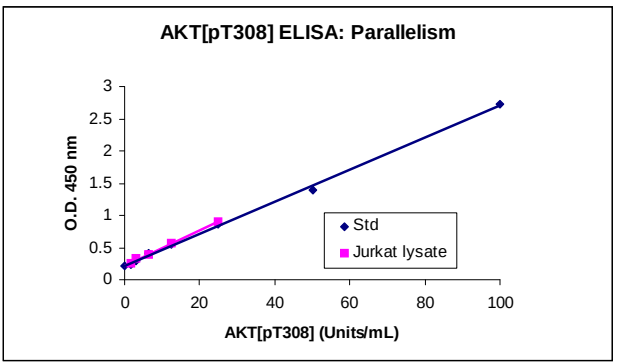
RECOVERY

To evaluate recovery, extract buffer was diluted 1:10 with *Standard Diluent Buffer* to bring the SDS concentration to <0.01%. Recombinant AKT [pT308] at 3 levels was spiked into the cell extract and percent recovery calculated over endogenous levels. On average, 102% recovery was observed.

PARALLELISM

Natural AKT [pT308] from extracts of Jurkat cells cultured in RPMI + 10% FCS were serially diluted in *Standard Diluent Buffer*. The optical density of each dilution was plotted against the AKT [pT308] standard curve. Parallelism demonstrated in Figure 2 indicates that the standard accurately reflects full length AKT [pT308] content in samples.

Figure 2



LINEARITY OF DILUTION

Extract Buffer was spiked with AKT [pT308] and serially diluted in *Standard Diluent Buffer* over the range of the assay. Linear regression analysis of sample values versus the expected concentration yielded a correlation coefficient of 0.99.

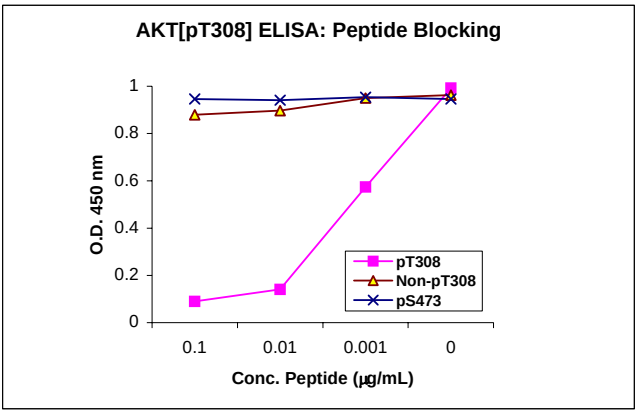
	Extract Buffer		
Dilution	**Measured (Units/mL)**	**Expected (Units/mL)**	**% Expected**
Neat	75	75	100%
1/2	38.5	37.5	103%
1/4	21.7	18.8	116%
1/8	11.9	9.4	126%

SPECIFICITY

The specificity of this assay for AKT phosphorylated at threonine 308 was confirmed by peptide competition. The data presented in Figure 3 show that only the phospho-peptide containing the phosphorylated threonine could block the ELISA signal. The same sequence containing non-phosphorylated threonine at position 308 did not block the signal.

Also, the assay was found to have no cross-reactivity with the following recombinant phosphoproteins tested at 100 ng/mL: p38 MAPK, p44 ERK1, p42 ERK2, JNK1, human insulin receptor, rat insulin receptor, human HGFR (c-met).

Figure 3



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Explanation of symbols

Symbol	Description	Symbol	Description
	Catalogue Number		Batch code
	Research Use Only		*In vitro* diagnostic medical device
	Use by		Temperature limitation
	Manufacturer		European Community authorised representative
[-]	Without, does not contain	[+]	With, contains
	Protect from light		Consult accompanying documents
	Directs the user to consult instructions for use (IFU), accompanying the product.		

AKT [pT308] Assay Summary