invitrogen

ELISA Kit
Catalog #KHO5421

p21 Waf1/Cip1

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INTRODUCTION

p21 Waf1/Cip1 is a 164 amino acid protein with the molecular weight of 21 kDa. The expression of p21 Waf1/Cip1 is p53 dependent. It may also be up-regulated independently of p53 by TGF-β, progesterone, nerve growth factor and apicidin. The stability of p21 Waf1/Cip1 protein is regulated by proteosomal degradation.

p21 Waf1/Cip1 plays important roles in several cellular pathways in response to intracellular and extracellular stimuli. In particular, it is involved in growth arrest induced by cell-cycle checkpoints, senescence, or terminal differentiation. Cell division relies on the activation of cyclins, which bind to CDKs to induce cell-cycle progression towards S phase and later to initiate mitosis. Their function is tightly regulated by cell-cycle inhibitors such as p21 Waf1/Cip1 and p27Kip1 proteins. Following anti-mitogenic signals or DNA damage, p21 Waf1/Cip1 and p27Kip1 bind to cyclin–CDK complexes to inhibit their catalytic activity and induce cell cycle arrest.

p21 Waf1/Cip1 regulates the activity of NF-kB, STAT3, Myc, C/EBP and E2F, and its expression inhibits several genes involved in cell cycle progression such as those encoding DNA polymerase, topoisomerase II, cyclin B1 and cdk1. p21 Waf1/Cip1 interacts with the histone acetylase CBP/p300, suggesting that it might function as a global regulator of CBP-dependent promoters through the regulation of CBP histone acetylase activity or its contact with RNA polymerases. p21Waf1/Cip1 functions as a transcriptional repressor when artificially tethered to DNA. Through E2F, myc and STAT3, p21 Waf1/Cip1 may down-regulate genes more specifically involved in mitogenic responses.

The Invitrogen p21 Waf1/Cip1 ELISA kit is designed to detect and quantify the level of p21 Waf1/Cip1 protein. This assay is intended for the detection of human p21 Waf1/Cip1 protein from cell lysates.

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READ ENTIRE PROTOCOL BEFORE USE

PRINCIPLE OF THE METHOD

The Invitrogen p21 Waf1/Cip1 kit is a solid phase sandwich <u>E</u>nzyme <u>L</u>inked <u>I</u>mmuno-<u>S</u>orbent <u>A</u>ssay (ELISA). A monoclonal antibody specific for human p21 Waf1/Cip1 has been coated onto the wells of the microtiter strips provided. Samples, including a standard of p21 Waf1/Cip1, control specimens, and unknowns, are pipetted into these wells. During the first incubation, the p21 Waf1/Cip1 antigen binds to the immobilized (capture) antibody. After washing, a rabbit antibody specific for p21 Waf1/Cip1 is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized p21 Waf1/Cip1 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 p21 Waf1/Cip1 present in the original specimen.

REAGENTS PROVIDED

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

Reagent	*96 Test Kit*
P21 Waf1/Cip1 Standard: Contains 0.1% sodium azide. Refer to vial label for quantity and reconstitution volume.	2 vials
Standard Diluent Buffer. Contains 0.1% sodium azide; red dye*; 25 mL per bottle.	1 bottle
*Antibody Coated Well*s, 12x8 Well Strips.	1 plate
p21 Waf1/Cip1 Detection Antibody. Contains 0.1% sodium azide; blue dye*; 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; yellow dye*; 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
* In order to help our customers avoid any mistakes in pipetting the ELISAs, we provide colored *Standard Diluent Buffer*, *Detection Antibody*, and *HRP Diluent* to help monitor the addition of solution to the reaction well. This does not in any way interfere with the test results.	

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 that are greater than the highest standard point should be diluted with *Standard Diluent Buffer* and retested.
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 500 μl per 5 ml Cell Extraction Buffer.

This buffer is stable for 2 to 3 weeks at $4^{o}C$ or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at $-20^{o}C$. When stored frozen, the Cell Extraction Buffer should be thawed on ice. This buffer (minus protease inhibitor cocktail and PMSF) can be obtained from Invitrogen Cat. # FNN0011. **Important:** add the protease inhibitors just before using. The stability of protease inhibitor supplemented Cell Extraction Buffer is 24 hours at $4^{o}C$. PMSF is very unstable and must be added prior to use, even if added previously.

B. Protocol for Cell Extraction

This protocol has been successfully applied to several cell lines. 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 p21 Waf1/Cip1. For example, 10^8 MCF-7 cells can be extracted in 1 ml of Extraction Buffer. Under these conditions, use of 1-10 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 p21 Waf1/Cip1.
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 p21 Waf1/Cip1 Standard

Note: This *p21 Waf1/Cip1 Standard* is prepared using purified, full length, recombinant p21 Waf1/Cip1 protein expressed in *E. coli*.

1. Reconstitute *p21 Waf1/Cip1 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 1000 pg/ml p21 Waf1/Cip1. Use the standard within 1 hour of reconstitution.
2. Add 0.25 ml of *Standard Diluent Buffer* to each of 6 tubes labeled 500, 250, 125, 62.5, 31.25, and 15.7 pg/ml p21 Waf1/Cip1.
3. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

B. Dilution of p21 Waf1/Cip1 Standard

Standard:	Add:	Into:
1000 pg/ml	Prepare as described in step 1	
500 pg/ml	0.25 ml of the 1000 pg/ml std.	0.25 ml of the Diluent Buffer
250 pg/ml	0.25 ml of the 500 pg/ml std.	0.25 ml of the Diluent Buffer
125 pg/ml	0.25 ml of the 250 pg/ml std.	0.25 ml of the Diluent Buffer
62.5 pg/ml	0.25 ml of the 125 pg/ml std.	0.25 ml of the Diluent Buffer
31.25 pg/ml	0.25 ml of the 62.5 pg/ml std.	0.25 ml of the Diluent Buffer
15.7 pg/ml	0.25 ml of the 31.25 pg/ml std.	0.25 ml of the Diluent Buffer
0 pg/ml	0.25 ml of the Diluent Buffer	An empty tube

Remaining reconstituted standard should be discarded or frozen in aliquots at -80°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. 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, controls, and diluted samples (>1:10 dilution for cell extract) to the appropriate microtiter wells. 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.**

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 *p21 Waf1/Cip1 Detection Antibod*y 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 p21 Waf1/Cip1 concentrations for unknown samples and controls from the standard curve plotted in step 16. **Multiply value(s) obtained for sample(s) by the appropriate dilution factor to correct for the dilution with *Standard Diluent Buffer*.** (Samples producing signals higher than the highest standard [1000 pg/ml] should be further diluted in *Standard Diluent Buffer* and reanalyzed, multiplying the concentration by the appropriate dilution factor.)

TYPICAL DATA

The following data were obtained for the various standards over the range of 0 to 1000 pg/ml p21 Waf1/Cip1.

Standard p21 Waf1/Cip1 (pg/ml)	Optical Density (450 nm)
1000	3.305
500	2.196
250	1.099
125	0.598
62.5	0.334
31.25	0.208
15.7	0.160
0	0.097

LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the 1000 pg/ml standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute samples >1000 pg/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 p21 Waf1/Cip1 in various matrices has not been investigated. Although p21 Waf1/Cip1 degradation 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 <5 pg/ml of p21 Waf1/Cip1. 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 p21 Waf1/Cip1. The data presented in Figure 1 show that the sensitivity of the ELISA is approximately 4x greater than that of western blotting. The bands shown in the western blotting data were developed using mouse anti-p21 Waf1/Cip1 antibody, and an alkaline phosphatase conjugated anti-mouse IgG followed by chemiluminescent substrate and autoradiography.

Figure 1: Detection of p21 Waf1/Cip1 by ELISA vs Western Blot:

p21 Waf1/Cip1

21 kDa

MCF-7 cell lysate

µg/test	40	20	10	5	2.5	1.25	0.625	0

ELISA

O.D. (450 nm)	2.954	1.583	0.979	0.627	0.354	0.208	0.142	0.062
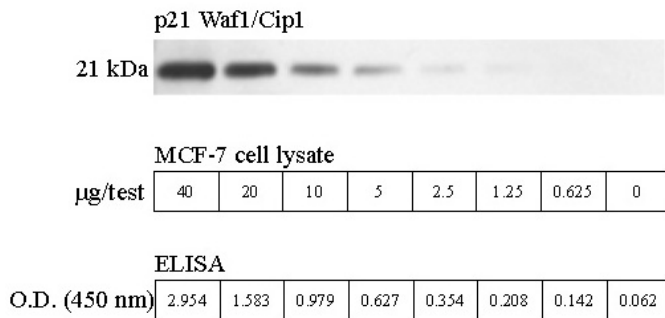
PRECISION

1. Intra-Assay Precision

Samples of known p21 Waf1/Cip1 concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (pg/ml)	756.38	224.50	84.78
SD	34.98	6.68	2.05
%CV	4.63	2.98	2.42

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 48 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (pg/ml)	714.53	225.00	81.08
SD	53.35	7.43	3.85
%CV	7.47	3.30	4.75

SD = Standard Deviation
CV = Coefficient of Variation

RECOVERY

To evaluate recovery, p21 Waf1/Cip1 standard was spiked at 3 different concentrations into 5% Cell Extraction Buffer. The average recovery was 86%.

PARALLELISM

Natural p21 Waf1/Cip1 from MCF-7 cell lysate was serially diluted in *Standard Diluent Buffer*. The optical density of each dilution was plotted against the p21 Waf1/Cip1 standard curve. Parallelism demonstrated by the figure below indicated that the standard accurately reflects p21 Waf1/Cip1 content in samples.

Figure 2. Parallelism between standard and natural cell lysates
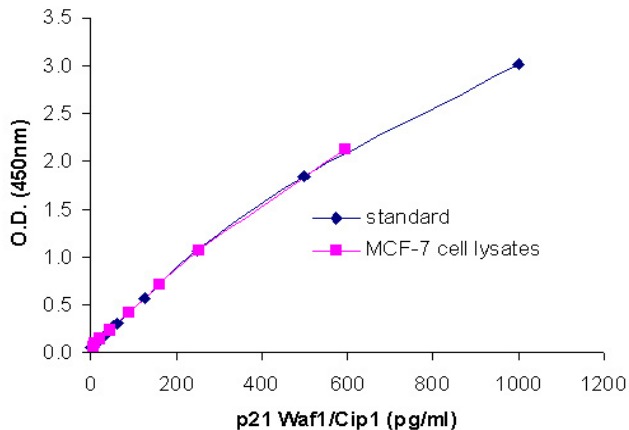
LINEARITY OF DILUTION

MCF-7 cells were grown in tissue culture medium containing 10% fetal bovine serum and lysed with Cell Extraction Buffer. This lysate was diluted in *Standard Diluent Buffer* over the range of the assay and measured for p21 Waf1/Cip1. Linear regression analysis of sample values versus the expected concentration yielded a correlation coefficient of 0.99.

Dilution	Cell Lysate		
	Measured (pg/ml)	Expected (pg/ml)	% Expected
neat	595.76	595.76	100.00
1/2	253.27	297.88	85
1/4	161.36	148.94	108
1/8	89.72	74.47	120
1/16	43.48	37.24	117

SPECIFICITY

The p21 Waf1/Cip1 ELISA recognizes human p21 Waf1/Cip1 and does not cross react with mouse and rat p21 Waf1/Cip1. Other species have not been tested.

In Figure 3, MCF-7 cells were treated with 2 μg/ml apicidin for 24 hours. Untreated MCF-7 cells were used as control. Cell extracts were prepared and cell lysates (200 μg/ml) were analyzed with p21 Waf1/Cip1 ELISA kit (Cat. # KHO5421). The data show that apicidin increases p21 Waf1/Cip1 expression level in MCF-7 cells. The data are representative of three independent experiments.

Figure 3. p21 Waf1/Cip1 expression in MCF-7 cells
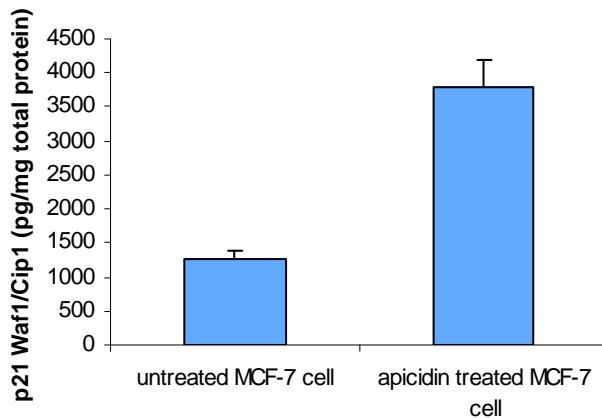
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Explanation of symbols

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

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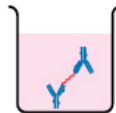
NOTES

P21 Waf1/Cip1 Assay Summary

Incubate 100 μl Standard or Cell Extract (>1:10 dilution) for 2 hours at RT
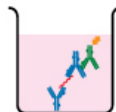
aspirate and wash 4x
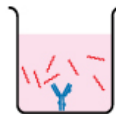
Incubate 100 μl of Detection Antibody for 1 hour at RT
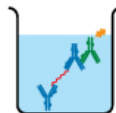
aspirate and wash 4x

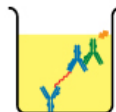Incubate 100 μl of HRP Anti-Rabbit Antibody for 30 minutes at RT

aspirate and wash 4x

Incubate 100 μl of Stabilized Chromogen for 30 minutes at RT

Add 100 μl of Stop Solution and read at 450 nm

Total time: 4 hours

p21 Waf1/Cip1 — HRP Anti-Rabbit Antibody — Detection Antibody