Enzo

Product Manual

p21 (human), ELISA kit

Catalog # ADI-900-161

96 Well Enzyme Immunoassay Kit

For use with cell lysates

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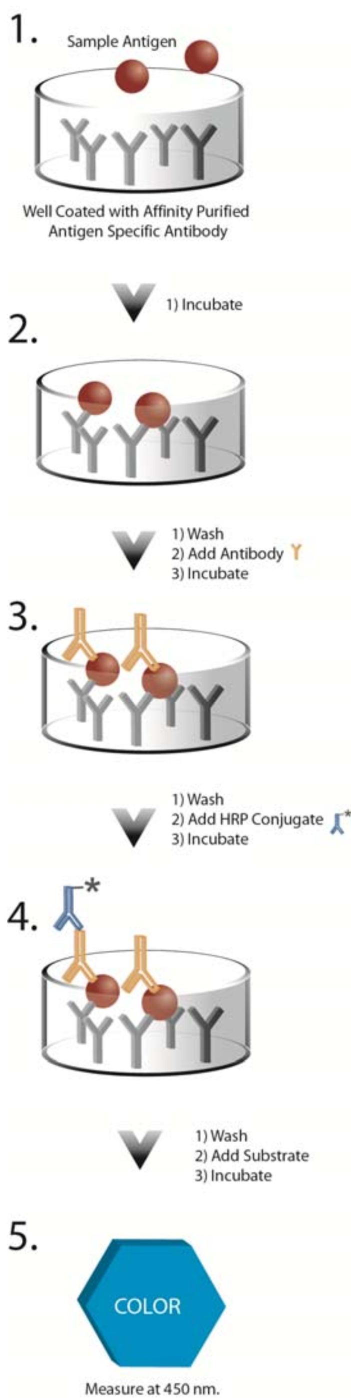
Check our website for additional protocols, technical notes, MSDS and FAQs.

For proper performance, use the insert provided with each individual kit received.

TABLE OF CONTENTS

Introduction 2
Principle 2
Materials Supplied 3
Storage 4
Other Materials Needed 4
Reagent Preparation 5
Sample Handling 6
Sample Recoveries 6
Assay Procedure 7
Calculation of Results 8
Typical Results 9
Performance Characteristics 10
References 12
Limited Warranty 14



INTRODUCTION

The p21 (human), ELISA kit is a complete kit for the quantitative determination of human p21 in cell lysates. Please read the complete kit insert before performing this assay.

Progression through the cell cycle is controlled by cyclin-dependent kinases (CDKs). Human p21WAF1/CIP1 is a CDK inhibitor that was first cloned and characterized as a mediator of p53-induced growth arrest[1-3]. Human p21 is a 164 amino acid nuclear protein with a calculated molecular weight of 18,119 Daltons whose expression is p53-dependent[4]. Human p21 is found in a complex involving cyclins, CDKs and PCNA in normal cells but not transformed cells. Human p21 may also be upregulated independently of p53 by TGF-β, tamoxifin, progesterone, nerve growth factor and apicidin[5-9]. Human p21 regulates cell cycle progression, terminal differentiation and apoptosis[10]. Human p21 expression has been implicated in a variety of human cancers including those of the prostate, bladder and esophagus[11-13]. Increased expression of the HOXB4 transcription factor in combination with suppression of p21 expression could be a useful strategy for effective and robust expansion of hematopoetic stem cells[14].

PRINCIPLE

1. Samples and standards are added to wells coated with a monoclonal antibody specific for human p21. The plate is then incubated.
2. The plate is washed, leaving only bound human p21 on the plate. A yellow solution of polyclonal antibody to human p21 is then added. This binds the human p21 captured on the plate. The plate is then incubated.
3. The plate is washed to remove excess p21 antibody. A blue solution of HRP conjugate is added to each well, binding to the human p21 polyclonal antibody. 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 human p21 in the sample

MATERIALS SUPPLIED

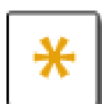
Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.

1. Assay Buffer 23 Concentrate (5x)

30mL, Catalog No. 80-1601
Tris buffered saline containing detergents.

2. human p21 Standard, 0.1 mL

Catalog No. 80-1205
One vial containing 40,000 pg/mL of recombinant human p21.



The standard should be handled with care due to the known and unknown effects of the antigen.

3. RIPA Cell Lysis Buffer 2

100 mL, Catalog No. 80-1284
50mM Tris HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 1% Sodium deoxycholate, and 0.1% SDS.

4. human p21 Clear Microtiter Plate

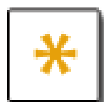
One Plate of 96 Wells, Catalog No. 80-1191
A plate of break-apart strips coated with a mouse monoclonal antibody specific to human p21.

5. Wash Buffer Concentrate (10X)

100 mL, Catalog No. 80-1287
Tris buffered saline containing detergents.

6. human p21 ELISA Antibody

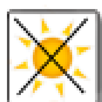
10 mL, Catalog No. 80-1193
A yellow solution of polyclonal antibody to human p21.



Activity of conjugate is affected by nucleophiles such as azide, cyanide, and hydroxylamine.

7. human p21 ELISA Conjugate

10 mL, Catalog No. 80-1192
A blue solution of goat anti-rabbit IgG conjugated to horseradish peroxidase.



Protect substrate from prolonged exposure to light.

8. TMB Substrate

10 mL, Catalog No. 80-0350
A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide.

Stop solution is caustic. Keep tightly capped.

9. Stop Solution 2

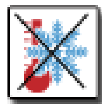
10 mL, Catalog No. 80-0377
A 1N solution of hydrochloric acid in water.

10. human p21 Assay Layout Sheet

1 each, Catalog No. 30-0237

11. Plate Sealer

3 each, Catalog No. 30-0012



Reagents require separate storage conditions.

STORAGE

All components of this kit, except the human p21 Standard, are stable at 4°C until the kit's expiration date. The human p21 Standard must be stored at -20°C .

OTHER MATERIALS NEEDED

1. Deionized or distilled water.
2. Precision pipets for volumes between 50 µL and 1,000 µL.
3. Repeater pipet for dispensing 100 µL.
4. Disposable beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Lint-free paper for blotting.
8. Microplate reader capable of reading at 450 nm.
9. Graph paper for plotting the standard curve.
10. Disposable polypropylene tubes for dilution of samples and standards.
11. Phenylmethylsulfonylfluoride (PMSF), Sigma P7626 or equivalent.
12. Protease inhibitor cocktail (PIC), Sigma P1860 or any other PIC **not** containing AEBSF.

Bring all reagents to room temperature for at least 30 minutes prior to opening.

Plastic tubes must be used for standard preparation.

REAGENT PREPARATION

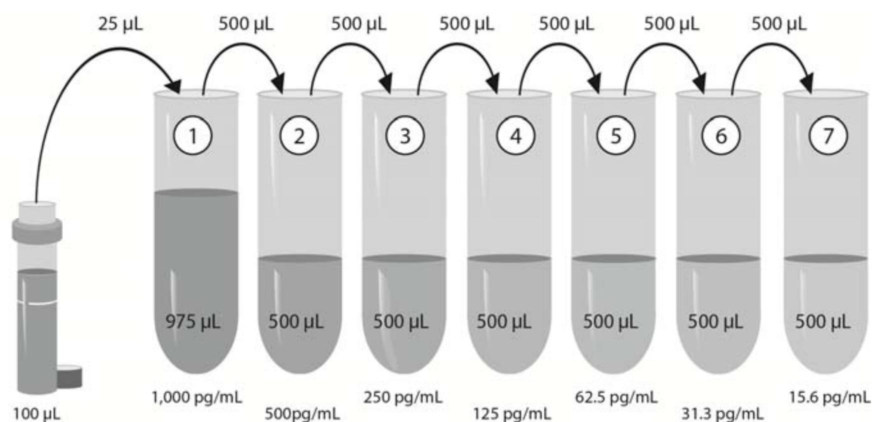
1. **Wash Buffer**

   Prepare the wash buffer by diluting 50 mL of the supplied Wash Buffer 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. **2. human p21 Standards**

   Allow the 40,000 pg/mL human p21 standard solution to warm to room temperature. Label seven 12 x 75 mm polypropylene tubes #1 through #7. Pipet 975 µL of Assay Buffer 23 plus inhibitors into tube #1. Pipet 500 µL of Assay Buffer 23 plus inhibitors into tubes #2 through #7. Add 25 µL of the 40,000 pg/mL standard into tube #1 and vortex thoroughly. 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.

   The concentration of human p21 in tubes #1 through #7 will be 1000, 500, 250, 125, 62.5, 31.25, and 15.62 pg/mL respectivly.



   Diluted standards should be used within 30 minutes of preparation. The concentration of human p-21 in tubes is labeled above.

3. **Assay Buffer 23**

   Prepare the Assay Buffer 23 by diluting 25 mL of the supplied concentrate with 100 mL of deionized water. This can be stored at room temperature until the kit's expiration or 3 months, whichever is earlier.

4. **PIC and PMSF Addition**

   Immediately prior to use, PIC and PMSF must be added to the assay buffer and RIPA Cell Lysis Buffer 2. If using Sigma Protease Inhibitor Cocktail P1860 add 0.5 µL/mL or the equivalent concentration of AEBSF-free PIC according to the alternate vendors specification sheet. Add PMSF, such as Sigma P7626, to a final concentration of 1mM.

   Each inhibitor treated buffer should incubate for 5-10 minutes at room temperature prior to use. Buffers treated with inhibitors should be used within 1 hour of preparation.

SAMPLE HANDLING

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

The The p21 (human), ELISA kit is compatible with human p21 samples in a wide range of cell lysates. Prior to assay, frozen samples should be brought slowly to 4°C (on ice) and centrifuged, if necessary, to isolate residual cell debris.

Human p21 is susceptible to proteases and it is crucial to prevent contamination of the reagents.

Samples must be stored at or below -20°C to avoid loss of bioactive analyte. Avoid repeated freeze/ thaw cycles.

A minimum 1:40 dilution is required for RIPA Cell Lysis Buffer 2 to remove matrix interference of this buffer. Due to differences in cell types, number of cells, or total cellular protein concentration, lysates may require a greater dilution with assay buffer plus inhibitors to remove interference or to be read within the standard range.

SAMPLE RECOVERIES

Human p21 concentrations were measured in HeLa cell lysates. Human p21 was spiked into the undiluted samples of p21 HeLa cell lysate, diluted, and assayed in the kit. The following results were obtained:

Sample	Cells per mL of Lysate Buffer	Recovery (%)	Recommended Dilution
HeLa lysate	($4x10^6$ cells/mL)	96	$\geq$ 1:40

Bring all reagents to room temperature for at least 30 minutes prior to opening.

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 the antibody, conjugate and substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in assay results.

ASSAY PROCEDURE

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 the standard diluent into the S0 (0 pg/mL standard) wells.
2. Pipet 100 µL of Standards #1 through #7 to the bottom of the appropriate wells.
3. Pipet 100 µL of the samples to the bottom of the appropriate wells.
4. Seal the plate. Incubate for 1 hour on a plate shaker (~500 rpm) at room temperature.
5. Empty the contents of the wells and wash by adding 400 µL of wash buffer to every well. Repeat 4 more times for a total of 5 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 (~500 rpm) at room temperature.
14. Pipet 100 µL of stop solution into 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.

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 human p21 in 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 OD for each standard and sample by subtracting the average blank OD from the average OD for each standard and sample.

Average Net OD = Average OD - Average Blank OD

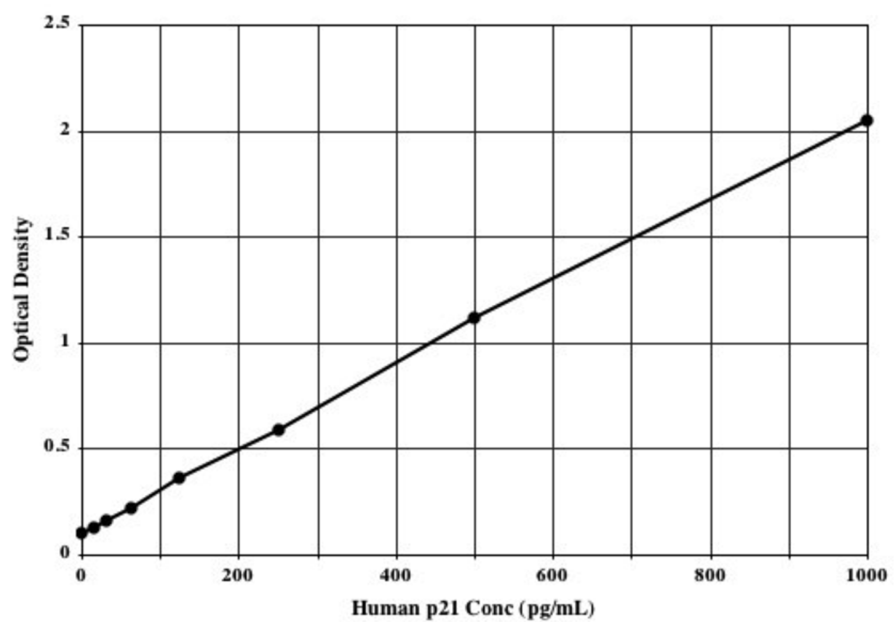
2. Plot the average Net OD for each standard versus human p21 concentration in each standard. Approximate a straight line through the points. The concentration of the unknowns can be determined by interpolation.

Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

TYPICAL RESULTS

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

Sample	Net OD	human p21 (pg/mL)
S0	0.102	**0**
S1	2.045	**1,000**
S2	1.113	**500**
S3	0.585	**250**
S4	0.362	**125**
S5	0.221	**62.5**
S6	0.157	**31.2**
S7	0.129	**15.6**
Unknown 1	1.156	**523.3**
Unknown 2	0.183	**43.6**



PERFORMANCE CHARACTERISTICS

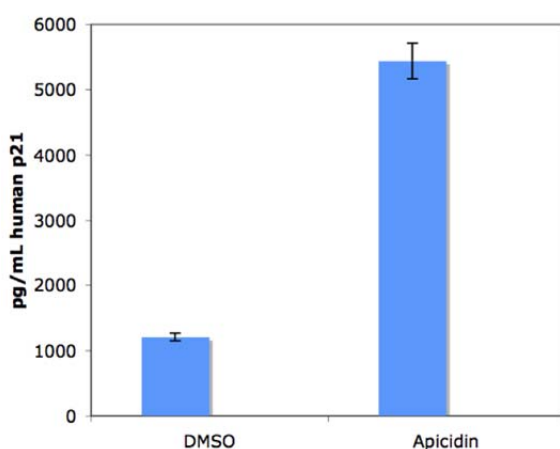
Specificity

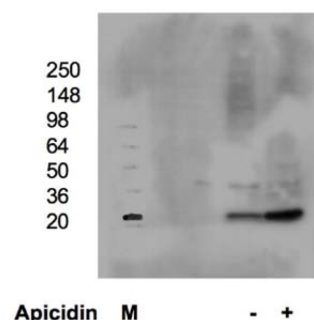
The cross reactivities for a number of related compounds were determined by diluting potential cross reactants in Assay Buffer 23 at 100,000 pg/mL. These samples were then measured in the assay.

Compound	Cross Reactivity
cIAP1	< .02%
p38	< .02%
pMEK	< .02%
JNK	< .02%
β - Catenin	< .02%
p300	< .02%
Smac / DIABLO	< .02%

Stimulation Response

HeLa cells were stimulated with 2 µg/mL apicidin or DMSO for 24 hours. Cells were harvested, washed and cell pellets were resuspended with RIPA Cell Lysis Buffer 2 plus inhibitors at 4 x 10^6 cells/mL (~1 mg/mL total cellular protein). The cells were vortexed and lysed on ice for 30 minutes. The lysates were vortexed and pelleted at 16,000 x g for 20 minutes at 4°C. The supernatants were either assayed immediately by Western analysis and ELISA or stored at -70°C. The results of the Western blotting and ELISA are shown below. Apicidin treatment resulted in a 4.4 fold increase in p21 levels by ELISA that mirrors the increase observed by Western blotting.





Sensitivity

The sensitivity of the assay, defined as the concentration of human p21, measured at 2 standard deviations from the mean of 24 zeros along the standard curve, was determined to be 8.0 pg/mL.

Linearity

A lysate from apicidin treated HeLa cells was diluted 1:40 and then further diluted serially. The results are shown in the table below.

Dilution	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1:40	--------------	324 pg/mL	-----------
1:80	262 pg/mL	240 pg/mL	92 %
1:160	131 pg/mL	120 pg/mL	109 %
1:320	65.5 pg/mL	63.6 pg/mL	103 %
1:640	32.8 pg/mL	33.1 pg/mL	99 %

A buffer sample containing human p21 was serilly diluted 1:2 in kit Assay Buffeer 23 and measured in the assay. The results are shown in the table below.

Dilution	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
Neat	--------------	781 pg/mL	-----------
1:2	390 pg/mL	411 pg/mL	105 %
1:4	195 pg/mL	196 pg/mL	101 %
1:8	97.6 pg/mL	99.1 pg/mL	102 %
1:16	48.8 pg/mL	51.1 pg/mL	105 %
1:32	24.4 pg/mL	27.5 pg/mL	113 %

Precision

Intra-assay precision was determined by assaying 20 replicates of three buffer controls containing human p21 in a single assay.

pg/mL	%CV
538	5.3
200	2.1
46	17.2

Inter-assay precision was determined by measuring buffer controls of varying human p21 concentrations in multiple assays over several days.

pg/mL	%CV
528	8.1
201	8.8
45	13.3

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