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> p27/Kip1 (human), EIA kit

Catalog # ADI-900-139

96 Well Enzyme Immunoassay Kit
For use with cell lysates

All reagents, except standard, should be stored at 4˚C. Store standard at -20˚C.

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

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

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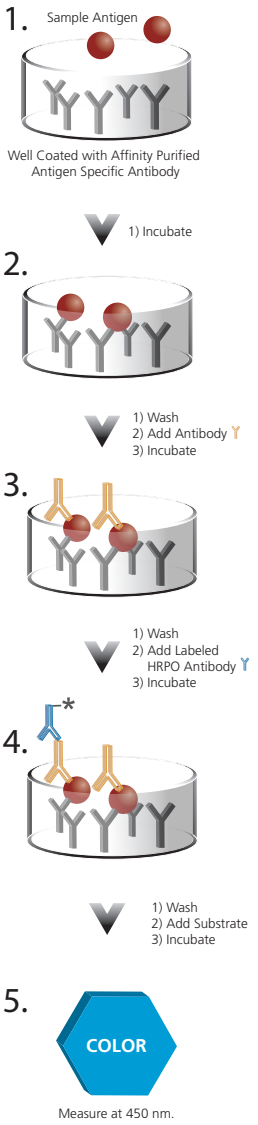


Introduction

The p27/Kip1 (human), EIA 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].

Principle

1. Samples and standards are added to wells coated with a monoclonal antibody specific for $p27^{Kip1}$. The plate is then incubated.

2. The plate is washed, leaving only bound $p27^{Kip1}$ on the plate. A yellow solution of polyclonal antibody to human $p27^{Kip1}$ is then added. This binds the $p27^{Kip1}$ 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 $p27^{Kip1}$ 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 $p27^{Kip1}$ in the sample

Materials Supplied

1. RIPA Cell Lysis Buffer 3
   Two bottles, each containing, 105 mL, Catalog No. 80-1496
   50mM Tris HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1.2% Triton X-100, 0.5% Sodium Deoxycholate, and 0.1% SDS.

2. human $p27^{Kip1}$ Standard
   0.150 mL, Catalog No. 80-1313
   A solution of 64,000 pg/mL of recombinant human $p27^{Kip1}$.

3. $p27^{Kip1}$ Clear Microtiter Plate
   One Plate of 96 Wells, Catalog No. 80-1312
   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
   105 mL, Catalog No. 80-1287
   Tris buffered saline containing detergents.

5. Total $p27^{Kip1}$ EIA Antibody
   11 mL, Catalog No. 80-1317
   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, Catalog No. 80-1318
   A blue solution of goat anti-rabbit IgG conjugated to horseradish peroxidase.

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

8. Stop Solution 2
   11 mL, Catalog No. 80-0377
   A 1N solution of hydrochloric acid in water.

9. human Total $p27^{Kip1}$ Assay Layout Sheet
   1 each, Catalog No. 30-0215

10. Plate Sealer
    3 each, Catalog No. 30-0012

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

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

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

Protect substrate from prolonged exposure to light.

Stop solution is caustic. Keep tightly capped.

Storage

Reagents require separate storage conditions.

All components of this kit, except the standard, are stable at 4°C untill the kit's expiration date. The standard **must** be stored at or below -20°C.

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.

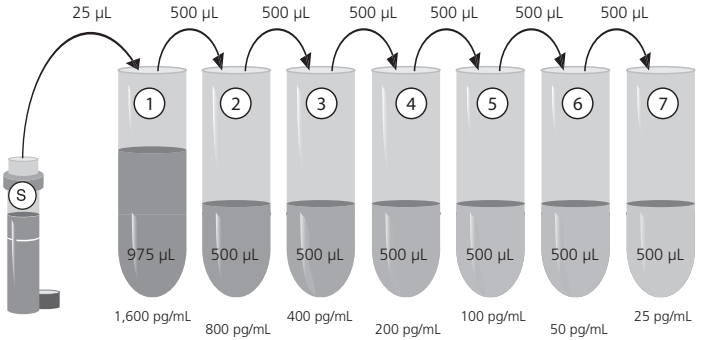
Reagent Preparation

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. **PIC Addition**
   Immediately prior to use, PIC must be added to the RIPA Cell Lysis Buffer 3. If using Sigma Protease Inhibitor Cocktail #P8340, add 0.5 µL/mL PIC, or equivalent concentration according to alternate vendor's specification sheet.

   Inhibitors must be freshly added to the RIPA Cell Lysis Buffer 3 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.

3. **human p27^{Kip1} Standards**



Label seven 12 x 75 mm polypropylene tubes #1 through #7. Pipet 975 µL of the assay buffer into tube #1. Pipet 500 µL of the assay buffer into tubes #2 through #7. Add 25 µL of the 64,000 pg/mL standard stock 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.

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 minumum time possible.

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

Bring all reagents, except RIPA Cell Lysis Buffer 3, to room temperature for at least 30 minutes prior to opening. RIPA Cell Lysis Buffer 3 is best used cold.

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.

Plastic tubes must be used for standard preparation.

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

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 4ºC 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 3 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

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 4ºC. Discard supernatant.
2. Resuspend pellet and wash with Hank's Balanced Salt Solution.
3. Centrifuge at 7,000 rpm for 10 minutes at 4ºC. 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 4ºC. The supernatants can be stored at or below -20ºC or used immediately in the assay.

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 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 buffer to every well. Repeat 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 (~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.

Bring all reagents, except RIPA Cell Lysis Buffer 3, to room temperature for at least 30 minutes prior to opening. RIPA Cell Lysis Buffer 3 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.

Calculation of Results

Several options are available for the calculation of the concentrations of p27^{Kip1} 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. Using linear graph paper, plot the average Net OD for each standard versus IGF-I concentration in each standard. Approximate a straight line through the points. The concentration of the unknowns can be determined by interpolation.

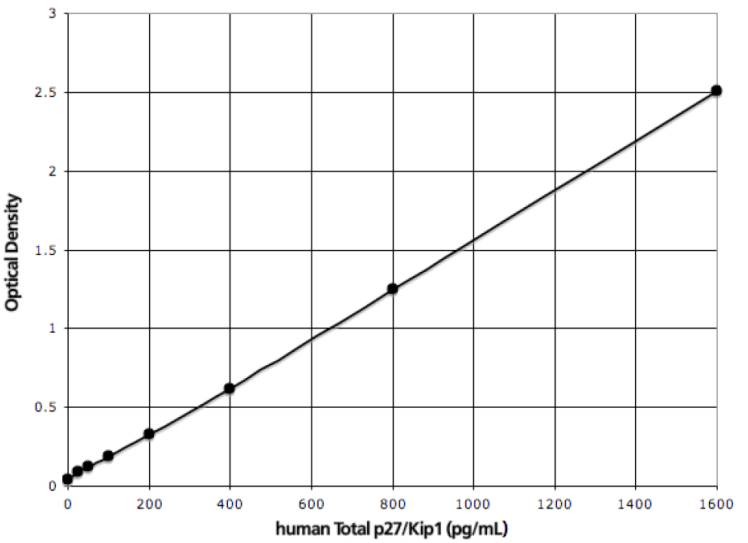
Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

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	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**



Performance Characteristics

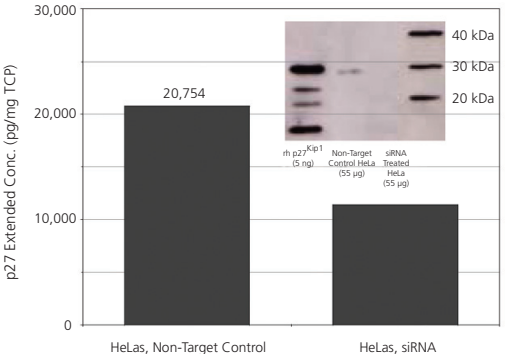
Specificity

The cross reactivities for a number of related compounds were determined by diluting 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%

Down Regulation Experiment

The numbers of HeLa cells lysed in this experiment were 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. 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.



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.

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 %

Precision

Intra-assay precision was determined by assaying 24 replicates of three buffer controls containing p27^{Kip1} 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 p27^{Kip1} concentrations in multiple assays over several days.

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

References

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MSDS (Material Safety Data Sheet) available online

Use of Product

This product contains research chemicals. As such, they should be used and handled only by or under the supervision of technically qualified individuals. This product is not intended for diagnostic or human use.

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