SunLong Biotech Co.,LTD
Tel: 0086-571-56623320 Fax: 0086-571-56623318
E-mail: sales@sunlongbiotech.com
www.sunlongbiotech.com

# Sunlong Medical™ Human P16-INK4A One-Step ELISA Kit

**Catalog Number:** One-EL197Hu
**Size:** 96 Test

**Storage:** 2-8℃/-20℃
**Validity Period: 2-8℃: 6 months; -20℃: 12 months**

**Sensitivity:** 0.03 ng/mL
**Assay range:** 0.47-30 ng/mL

For the quantitative detection of human P16-INK4A concentration in cell lysate.

This package insert must be read entirely before using this product. For proper performance, follow the protocol provided with each individual kit.

**FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

# TABLE OF CONTENTS

ASSAY PROCEDURE SUMMARY ..... 1

## Introduction

Description ..... 2

Principle of the Assay ..... 2

Limitations of the Procedure ..... 2

## General Information

Materials Provided ..... 2

Storage ..... 3

Other Supplies Required ..... 3

Precaution ..... 3

Technical Hints ..... 3

## Assay Protocol

Sample Collection and Storage ..... 4

Reagent Preparation ..... 4

Assay Procedure ..... 5

## Analysis

Calculation of Results ..... 6

Typical Data ..... 6

Sensitivity ..... 7

Precision ..... 7

Recovery ..... 7

Linearity ..... 7

Calibration ..... 7

Sample Values ..... 7

Specificity ..... 8

# ASSAY PROCEDURE SUMMARY

**1. Preset the layout of the microplate, including control group, standard group and sample group, take out the required number of microplate strips and return excess strips to the foil pouch containing the drying reagent pack and reseal; store at 4°C immediately. Microplate strips should be used in one week.**

↓

**2. Add 50 µL standard or sample to appropriate wells. To avoid high background always add samples or standards to the well before the addition of antibody cocktail.**

↓

**3. Add 50 µL 1X Antibody Cocktail solution (refer to Reagent Preparation 7.2) to each well. Seal plate with cover seal and incubate at 37°C on a microplate thermostatic shaker set at 400 rpm for 1 hour (incubate at 37°C for 2 hours is recommended if thermostatic shaker is not available).**

↓

**4. Wash: 1) Gently remove the cover seal. Discard the liquid from wells by aspirating or decanting. Remove any residual solution by tapping the plate a few times on fresh paper towels. 2) Wash 4 times with 1X Wash Buffer, using at least 350-400 µL per well. Following the last wash, firmly tap plates on fresh towels 10 times to remove residual Wash Buffer. Avoid getting any towel fibers in the wells or wells drying out completely.**

↓

**5. Add 100 µL TMB substrate solution to each well, protected from light. Incubate at 37°C on a microplate thermostatic shaker set at 400 rpm for 15 to 20 minutes. (Substrate Solution should remain colorless until added to the plate.)**

↓

**6. Add 100 µL Stop Solution to each well in the same order as addition of the TMB substrate. Note: Avoid skin and eye contact with the Stop solution.**

↓

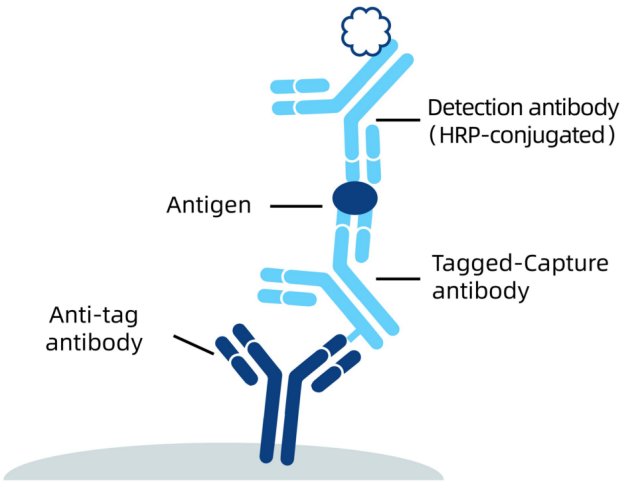
**7. Read results immediately on a microplate reader at a wavelength of 450 nm. If possible, perform a double wavelength readout (450 nm and 630 nm).**

## DESCRIPTION

CDKN2A encodes two transcripts with alternative transcriptional start sites. Both transcripts share exons 2 and 3, but are translated in different open reading frames to yield two distinct proteins: p16INK4a and ARF (p14ARF in humans, p19ARF in mice). The tumor suppressor protein p16Ink4a (encoded from the CDKN2A locus) is often transcriptionally activated in cells undergoing senescence and is one of the main regulators of this program, and it is upregulated in multiple tissues during aging. p16-Ink4a is the principal member of the Ink4 family of CDK inhibitors. p16-Ink4a contributes to the regulation of cell cycle progression by inhibiting the S phase. p16Ink4a binds to CDK4/6, inhibiting cyclin D-CDK4/6 complex formation and CDK4/6-mediated phosphorylation of Rb family members. Expression of p16-Ink4a maintains the Rb family members in a hypophosphorylated state, which promotes binding to E2F1 and leads to G1 cell cycle arrest.

## PRINCIPLE OF THE ASSAY

An anti-tag antibody is pre-coated onto the bottom of wells. After adding antigen or samples, Tagged-Capture antibody and HRP-conjugated detection antibody, a sandwich complex is formed in the solution. TMB acts as a HRP substrate, and the solution color will change from colorless to blue. A stop solution containing sulfuric acid turns the solution yellow. The color intensity is proportional to the quantity of bound protein, which is measurable at 450 nm with the correction wavelength set at 630 nm.




## LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use expired kit or reagents.
- Do not mix or substitute reagents or materials from other kit lots or other sources.
- If concentration of assayed factor in samples is higher than the highest standard, dilute the samples with the appropriate Sample Diluent and reanalyze.
- Any variation in testing personnel, sample preparation, standard dilution, pipetting technique, washing techniques, incubation time, temperature, kit age and equipment can cause variation in results.
- This assay is designed to eliminate interference by factors present in biological samples. Until all factors have been tested in the ELISA immunoassay, the possibility of interference cannot be excluded.

## MATERIALS PROVIDED (96 Test)

Unopened kit should be stored at 2 - 8°C.

- **Microplate:** 96 well microplate precoated an anti-tag antibody (8 well × 12 strips) (1 plate)

- **Protein standard:** 60 ng/bottle; lyophilized (2 bottles)
- **Capture antibody (100×):** 60 µL/vial* (1 vial)
- **Detection antibody, HRP-conjugated (100×):** 60 µL/vial* (1 vial)
- **Sample Diluent PT 4-eg:** 30 mL/bottle (1 bottle)
- **Detection Diluent:** 15 mL/bottle (1 bottle)
- **Wash Buffer Concentrate (20×):** 30 mL/bottle (1 bottle)
- **Extraction Reagent:** 15 mL/bottle (1 bottle)
- **Tetramethylbenzidine Substrate (TMB):** 12 mL/bottle (1 bottle)
- **Stop Solution:** 12 mL/bottle (1 bottle)
- Plate Cover Seals (4 pieces)

## STORAGE

Store kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2 to 8°C). Expiration date of the kit and reagents is stated on the labels.

Expiration date of the kit components can only be guaranteed if the components are stored properly.

| | Condition |
|---|---|
| Unopened kit | Store at 2 - 8°C for 6 months or -20°C for 12 months. (See expiration date on the label.) |
| Opened kit | All reagents stored at 2 - 8°C for 7 days. Please use a new standard for each assay. |

## OTHER SUPPLIES REQUIRED

- A microplate reader capable of measuring absorbance at 450 nm with the correction wavelength set at 630 nm.
- Calibrated, adjustable precision pipettes and disposable plastic tips. A manifold multi-channel pipette is recommended for large assays.
- Plate washer: automated or manual.
- Absorbent paper towels.
- Glass or plastic tubes to prepare standard and sample dilutions.
- Beakers and graduated cylinders.
- Log-log or semi-log graph paper or computer and software for ELISA data analysis. A four-parameter logistic (4-PL) curve-fit is recommended.
- Microplate thermostatic shaker.

## PRECAUTION

- Avoid any skin and eye contact with Stop Solution and TMB. In case of contact, wash thoroughly with water.
- Do not use the kit after the expiration date.
- Do not mix or substitute reagents or materials from other kit lots or other sources.
- Be sure to wear protective equipment such as gloves, masks and goggles during the experiment.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer to improve assay precision

## TECHNICAL HINTS

- Bring all reagents to room temperature before use.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

- When using an automated plate washer, adding a 30 second soak period before washing step and/or rotating the plate between wash steps may improve assay precision.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution.
- The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.
- It is recommended that all samples and standards be assayed in duplicate.

## SAMPLE COLLECTION AND STORAGE

### Cell Lysate

1) Collect cells and wash by centrifuging at 500 x g for 5 minutes before resuspension in pre-cooled PBS buffer. Perform this step three times. 2) Count cells and then discard the supernatant. 3) Add protease inhibitor cocktail to the Extraction Reagent to a final concentration immediately prior to performing cell lysis. 4) Add 1 mL of Extraction reagent (containing protease inhibitor cocktail) Per 1 x 107 cells, Incubate cell suspension on ice for 30 minutes, use ultrasound to treat the samples. 5) Centrifuge cell lysate at 10,000 x g for 5 minutes at 4℃. 6) Measure the concentration of total protein in cell lysate using BCA assay. Where possible, keep samples on ice to avoid protein degradation.

## REAGENT PREPARATION

Bring all reagents and samples to room temperature before use. If crystals have formed in the Wash Buffer Concentrate, warm to room temperature and mix gently until the crystals have completely dissolved.

### Wash Buffer (1X)

If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 30 mL of Wash Buffer Concentrate(20X) to 570 mL deionized or distilled water to prepare 1X Wash Buffer.

### Antibody Cocktail (1X)

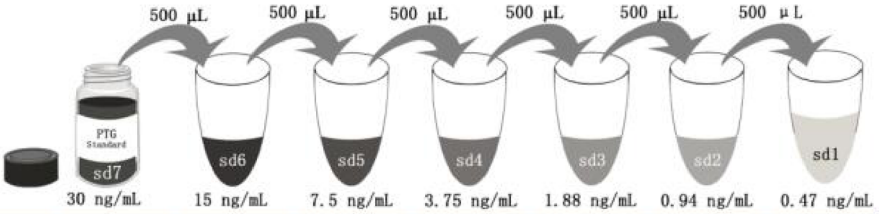
Dilute 100X capture antibody and 100X HRP-conjugated detection antibody using Detection Diluent prior to assay. Suggested 1:100 dilution: 50 µL 100X capture antibody + 50 µL 100X Detection Antibody, HRP-conjugated + 4,900 µL Detection Diluent. Mix gently but thoroughly.

### Sample Dilution

Different samples should be diluted with corresponding Sample Diluent, samples may require further dilution if the readout values are higher than the highest standard OD reading. Variations in sample collection, processing and storage may affect the results of the measurement. Recommended Dilution for different sample types: 1:20 or 1:40 is recommended for cell lysate.

### Standard Serial Dilution

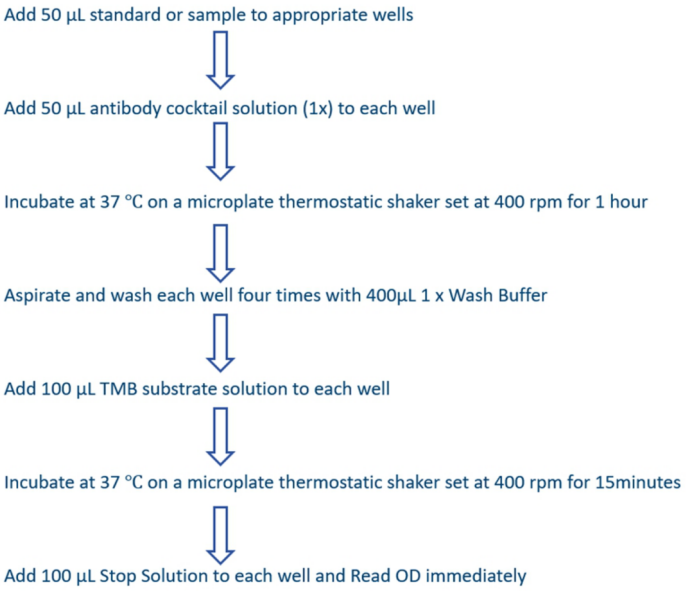
Add 2 mL Sample Diluent PT 4-eg in protein standard.




| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Add # µL of Standard diluted in the previous step | — | 500 µL | 500 µL | 500 µL | 500 µL | 500 µL | 500 µL |
| # µL of Sample Diluent PT 4-eg | **2000 µL** | 500 µL | 500 µL | 500 µL | 500 µL | 500 µL | 500 µL |
| | "sd7" | "sd6" | "sd5" | "sd4" | "sd3" | "sd2" | "sd1" |

## ASSAY PROCEDURE

Bring all reagents to room temperature before use (Detection antibody, HRP-conjugated antibody can be used immediately). To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.




**1.** Preset the layout of the microplate, including control group, standard group and sample group, take out the required number of microplate strips and return excess strips to the foil pouch containing the drying reagent pack and reseal; store at 4°C immediately. Microplate strips should be used in one week.

**2.** Add 50 µL standard or sample to appropriate wells. To avoid high background always add samples or standards to the well before the addition of antibody cocktail.

**3.** Add 50 µL 1X Antibody Cocktail solution (refer to Reagent Preparation 7.2) to each well. Seal plate with cover seal and incubate at 37°C on a microplate thermostatic shaker set at 400 rpm for 1 hour (incubate at 37°C for 2 hours is recommended if thermostatic shaker is not available).

**4.** Wash: 1) Gently remove the cover seal. Discard the liquid from wells by aspirating or decanting. Remove any residual solution by tapping the plate a few times on fresh paper towels. 2) Wash 4 times with 1X Wash Buffer, using at least 350-400 µL per well. Following the last wash, firmly tap plates on

fresh towels 10 times to remove residual Wash Buffer. Avoid getting any towel fibers in the wells or wells drying out completely.

**5.** Add 100 µL TMB substrate solution to each well, protected from light. Incubate at 37°C on a microplate thermostatic shaker set at 400 rpm for 15 to 20 minutes. (Substrate Solution should remain colorless until added to the plate.)

**6.** Add 100 µL Stop Solution to each well in the same order as addition of the TMB substrate. Note: Avoid skin and eye contact with the Stop solution.

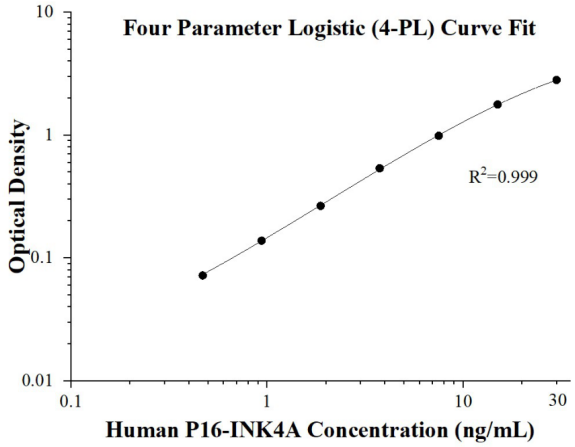
**7.** Read results immediately on a microplate reader at a wavelength of 450 nm. If possible, perform a double wavelength readout (450 nm and 630 nm).

## CALCULATION OF RESULTS

Data analysis: Calculate the average of the duplicate readings (OD value) for each standard and sample, and subtract the average of the zero standard absorbance. Construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis, four-parameter logistic curvefit (4-PL) analysis is recommend. If the samples have been diluted, the fitting result must be multiplied by the dilution factor used.

## TYPICAL DATA

These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.




| (pg/mL) | O.D. | Average | Corrected |
|---|---|---|---|
| 0 | 0.0602<br>0.0577 | 0.05895 | - |
| 0.47 | 0.1405<br>0.1217 | 0.1311 | 0.07215 |
| 0.94 | 0.1989<br>0.196 | 0.19745 | 0.1385 |
| 1.88 | 0.328<br>0.322 | 0.325 | 0.26605 |
| 3.75 | 0.5985<br>0.5946 | 0.59655 | 0.5376 |
| 7.5 | 1.0577<br>1.0376 | 1.04765 | 0.9887 |
| 15 | 1.8503<br>1.8233 | 1.8368 | 1.77785 |
| 30 | 2.8636<br>2.8813 | 2.87245 | 2.8135 |

## SENSITIVITY

The minimum detectable dose of human P16-INK4A is 0.03 ng/mL. This was determined by adding two standard deviations to the concentration corresponding to the mean O.D. of 20 zero standard replicates.

## PRECISION

Intra-assay Precision (Precision within an assay) Three samples of known concentration were tested 8 times on one plate to assess intra-assay precision. Inter-assay Precision (Precision between assays) Three samples of known concentration were tested in 16 separate assays to assess inter-assay precision.

### Intra-assay Precision

| Sample | n | Mean (ng/mL) | SD | CV% |
|---|---|---|---|---|
| 1 | 8 | 16.37 | 0.38 | 2.32 |
| 2 | 8 | 3.98 | 0.14 | 3.52 |
| 3 | 8 | 2.01 | 0.10 | 4.98 |

### Inter-assay Precision

| Sample | n | Mean (ng/mL) | SD | CV% |
|---|---|---|---|---|
| 1 | 16 | 16.35 | 0.46 | 2.81 |
| 2 | 16 | 4.05 | 0.13 | 3.21 |
| 3 | 16 | 2.10 | 0.13 | 6.19 |

## RECOVERY

The recovery of human P16-INK4A spiked to three different levels throughout the range of the assay in cell lysate was evaluated.

| Sample Type | | Average% of Expected | Range (%) |
|---|---|---|---|
| Cell lysate | 1:80 | 103 | 102-104 |
| | 1:160 | 104 | 101-109 |

## LINEARITY

To assess the linearity of the assay, samples were diluted with the appropriate Sample Diluent to produce samples with values within the dynamic range of the assay. (The cell lysate was initially diluted 1:10. )

| | | Cell lysate |
|---|---|---|
| 1:2 | Average% of Expected | 100 |
| | Range (%) | - |
| 1:4 | Average% of Expected | 100 |
| | Range (%) | 98-101 |
| 1:8 | Average% of Expected | 91 |
| | Range (%) | 88-94 |
| 1:16 | Average% of Expected | 77 |
| | Range (%) | 74-79 |

## CALIBRATION

This immunoassay is calibrated against a highly purified recombinant **human Human P16-INK4A One-Step ELISA Kit** produced at Sunlong Medical™.

## SAMPLE VALUES

Cell lysate

| | Human P16-INK4A (ng/mL) | Total protein (mg/mL) |
|---|---|---|
| Hela cell lysate | 92.40 | 1.20 |

## SPECIFICITY

This kit specifically recognizes native and recombinant human P16-INK4A.

## PLATE LAYOUT

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | | | | | | | | |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |