KAMIYA BIOMEDICAL COMPANY

Human PIG3 ELISA

For the *in vitro* quantitation of human p53 Inducible Gene 3 protein in cell lysates, tissue culture medium, serum and plasma

Cat. No. KT-075

For research use only, not for use in diagnostic procedures.

PRODUCT INFORMATION

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PRODUCT

The **K-ASSAY®** Human PIG3 ELISA is a non-isotopic immunoassay for the *in vitro* quantitation of human p53 Inducible Gene 3 protein in cell lysates, tissue culture medium, serum and plasma. For Research Use only, not for use in diagnostic procedures.

INTRODUCTION

The product of p53-inducible gene 3 (PIG3) is a cytoplasmic protein, which is induced by exposure to genotoxic agents before the onset of apoptosis during p53-mediated growth arrest. The induction of PIG3 is p53-dependent and occurs with delayed kinetics as compared with other p53 downstream targets, such as p21 and MDM2. When cells lines are treated with adriamycin, a DNA-damaging and apoptotic-inducing agent known to increase endogenous p53 levels, PIG3, like p21, was found to be strongly induced in the cell lines with wild-type p53 genes, but not in the cell with mutant p53. This observation supports the p53-dependence of PIG3 induction. When p53-mediated growth arrest is reversed, elevated levels of PIG3 are maintained even in cells that resumed cycling in the absence of ectopic p53 expression, suggesting that PIG3 is a long-lived reporter, which may be useful for detecting transient activation of p53.

The proline-rich region of p53 is required for PIG3 activation. p53 protein lacking this region (p53delta62-91) can still induce many p53-responsive genes but not PIG3. This p53 mutant induces growth arrest but not apoptosis. Some tumor-derived p53-mutants, especially M246I, retained the ability to activate transcription of MDM2 but specifically fail to induce the PIG3 promoter, thus resembling p53delta62-91. Further, p53delta62-91 and p53M246I are defective for induction of apoptosis. PIG3 shares significant homology to TED2, a plant NADPH oxidoreductase. Interestingly, TED2 is one of the few genes implicated in the apoptotic process necessary for the formation of plant meristems. The closest relative of PIG3 in mammals is an NADPH–quinone oxidoreductase that is a potent generator of ROS. Reactive oxygen species are powerful inducers of apoptosis. Expression profiles (SAGE-based) of p53-induced genes suggested that p53 might induce apoptosis by stimulating the production of ROS. Wild-type p53 is a tumor suppressor gene which can activate or repress transcription, as well as induce apoptosis. The proteins encoded by these genes then collectively increase the content of ROS, which in turn damage mitochondria. Leakage of calcium and proteinaceous components from damaged mitochondria then stimulate the caspases that are ubiquitously activated during the apoptotic process.

PRINCIPLE

The PIG3 ELISA is a “sandwich” enzyme immunoassay employing mouse monoclonal antibodies. A monoclonal antibody, specific for the human PIG3 protein, has been immobilized onto the surface of microtiter wells provided in the kit. The sample to be assayed and the biotinylated detector monoclonal antibody are pipetted into the wells and allowed to incubate for two hours, during which time any PIG3 present binds to the capture and detecting antibodies. Unbound material is washed away and horseradish peroxidase-conjugated streptavidin is added, which binds to the detector antibody.

The horseradish peroxidase catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) from a colorless solution to a blue solution (or yellow after the addition of stop reagent), the intensity of which is proportional to the amount of PIG3 protein in the sample. The colored reaction product is quantified using a spectrophotometer.

Quantitation is achieved by the construction of a calibration curve using known concentrations of PIG3 (provided lyophilized). By comparing the absorbance obtained from a sample containing an unknown amount of PIG3 with that obtained from the calibrators, the concentration of PIG3 in the sample can be determined.

COMPONENTS

Calibrators should be assayed in duplicate. A calibration curve must be performed on the same plate and at the same time as the samples. The PIG3 ELISA provides sufficient reagents to run two sets of calibration curves, and 41 samples (if

assayed in duplicate all at once using one calibration curve), or 34 samples (if assayed on two separate occasions using two calibration curves).

- Coated Microtiter Plate: 96 removable wells coated with PIG3 monoclonal antibody.
- PIG3 Calibrator: Two vials containing lyophilized PIG3 protein. *Reconstituted calibrators should be discarded after one use.*
- Detector Antibody: 6 mL, biotinylated monoclonal anti-human PIG3 antibody.
- Conjugate: 30 µL, 400X concentrated Streptavidin-Peroxidase Conjugate solution.
- Conjugate Diluent: 12 mL, buffer for dilution of 400X Conjugate.
- Substrate: 11 mL, chromogenic substrate (TMB).
- Sample Diluent: 20 mL, buffer used to dilute calibrators and samples.
- Plate Wash Concentrate: 40 mL, 50X concentrated solution of PBS and surfactant.
- Cell Resuspension Buffer: 25 mL, 50 mM Tris, containing 5 mM EDTA, adjusted to pH 8.0.
- Cell Lysis Buffer: 4.2 mL, one vial, use as directed in sample preparation section for the extraction of PIG3 from cell preparations.
- Stop Solution: 11 mL, 2.5 N sulfuric acid.
- Plate Sealers: to cover plates during incubations.

MATERIALS REQUIRED BUT NOT PROVIDED

- 2-20 µL, 20-200 µL, and 200-1,000 µL precision pipetters with disposable tips.
- Automated plate washer, wash bottle or multi-channel dispenser for washing.
- 2 liter graduated cylinder.
- De-ionized or distilled H_2O.
- 0.2 µm syringe filter and syringe.
- Spectrophotometer capable of measuring absorbance in 96-well plates using dual wavelength of 450/595 nm or 450/540 nm. A single wavelength of 450 nm can also be used.

PRECAUTIONS AND RECOMMENDATIONS

- **Store all kit components at 4°C.** Do not expose reagents to excessive light.
- Let the kit sit at room temperature for 30 minutes before use. Best results will be obtained using reagents at room temperature.
- Wear disposable gloves and eye protection.
- Do not use the kit beyond the expiration date.
- Always use clean well-rinsed glassware. Soap residue may compromise assay performance.
- Use only the microtiter wells provided with the kit.
- Don not mix reagent from different kits.
- Do not mouth pipette or ingest any of the reagents.
- The buffers and reagents used in this kit contain anti-microbial and anti-fungal reagents. Care should be taken to prevent direct contact with these products.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Human samples may be contaminated with infectious agents. Do not ingest, expose to open wounds, or breathe aerosols. Dispose of samples properly.

PROTOCOLS
Sample Preparation

Cell Lysate

Numerous extraction protocols can be used. The following protocol has been shown to work with a number of cell lines. It is provided as an example of a suitable extraction procedure, but should not be construed as necessarily being the method of choice. Users may wish to experiment with extraction procedures that work best in their laboratory.

1. For suspension cells, pellet by centrifugation, remove supernatant, resuspend with PBS and pellet by centrifugation. For attached cells, remove supernatant from cells (you may save the supernatant for testing in the ELISA). Wash cells once with PBS, harvest cells by scraping and gentle centrifugation.
2. Aspirate PBS leaving an intact cell pellet (at this point the cell pellet can be frozen at –80°C and lysed at a later date). We recommend for every 5 x 10^6 cells, resuspend the pellet in 1 mL of Cell Resuspension Buffer provided.
3. Add 20 µL of Cell Lysis Buffer provided for every 100 µL of cell suspension.
4. Incubate 30 minutes on ice with occasional vortexing.
5. Transfer extracts to microcentrifuge tubes and centrifuge for 5 minutes at 500 x g at 4°C.
6. Aliquot cleared lysate to clean microfuge tubes. The sample should be aliquotted to avoid multiple freeze/thaws. These samples are now ready for analysis according to the instructions provided in the Assay Procedure. Samples may be stored at –20°C for future testing in the PIG3 ELISA.

Samples found to contain greater than 15 ng/mL PIG3 (i.e., outside the range of the calibration curve) must be diluted with Sample Diluent (provided), so that the PIG3 concentration falls within the range spanned by the calibration curve, and assayed again.

Serum and Plasma

For best results, sera and plasma should be diluted 1:5 with sample diluent. To prepare sera; freshly drawn blood is incubated at 37°C for 30 minutes to allow clot to form. Loosen clot from the tube by ringing the side of the tube with a pasteur pipet. Incubate at 4°C overnight, allowing the clot to contract. The serum is separated by centrifugation at 10,000 x g for 10 minutes at 4°C. Human plasma may be assayed in the PIG3 ELISA. We have tested heparinized plasma and found that it performs well in the assay, other anti-coagulants have not been tested.

ASSAY PROCEDURE

The PIG3 ELISA is provided with removable strips of wells so the assay can be carried out on two separate occasions. **Since conditions may vary, a calibration curve must be determined each time the assay is performed.** Calibrators should be assayed in duplicate. Disposable pipette tips and reagent troughs should be used for all transfers to avoid cross-contamination of reagents or samples.

1. Remove the appropriate number of microtiter wells from the foil pouch. Return any unused wells to the foil pouch, reseal and store at 4°C. Let all other kit components sit at room temperature until used. *Best results will be obtained using reagents at room temperature.*
2. Prepare a working solution (1X) of Wash Buffer by adding 40 mL of the 50X concentrated solution (provided), to 1,960 mL of de-ionized water. Mix well.
3. Each time an assay is performed, reconstitute a Lyophilized Calibrator by carefully and accurately pipetting dH_2O and/or sample diluent, as described on the lyophilized PIG3 Calibrator vial label to give a concentration of 15 ng/mL. Let the reconstituted calibrator sit for 15 minutes at room temperature, with occasional swirling. Avoid excessive agitation of the calibrator. After reconstituting the PIG3 Calibrator it should be diluted with Sample Diluent. Obtain six tubes and label them 15, 6, 2.4, 0.96, 0.384 and 0 ng/mL. Add 300 µL of Sample Diluent into each tube except the 15 ng/mL tube (first tube) which gets “undiluted” reconstituted calibrator. Remove 500 µL from the original vial of lyophilized material and add it to the first tube. Remove 200 µL from the first tube (15 ng/mL) and add it to the second tube (6 ng/mL) and mix gently. Repeat this procedure until you reach the sixth tube (0.384 ng/mL). The last tube (0 ng/mL) should just be Sample Diluent. **Reconstituted calibrators should be discarded after one use.**
4. Prepare all samples (see pages 3 and 4). **A recommended starting dilution for all samples is a 1:5 dilution with sample diluent.**
5. Pipette 50 µL of the Detector Antibody into each well.
6. Add samples and each of the PIG3 calibrators (in duplicate) by pipetting 50 µL into appropriate wells using clean pipette tips for each sample.
7. Cover wells with a plate sealer and incubate at room temperature for 2 hours.
8. Wash wells 3 times with 1X Wash Buffer making sure each well is filled completely.
9. Dilute a sufficient amount of the 400X Conjugate 1:400 in Conjugate Diluent to provide 100 µL of 1X solution for each sample and calibrator well (For example: add 30 µL to 11,970 mL of Conjugate Diluent), mix gently. **Filter with a 0.2 µm syringe filter prior to use.** Filtering will reduce background.
10. Pipette 100 µL of the 1X Conjugate into each well, cover with a plate sealer and incubate at room temperature for 30 minutes. Discard any unused 1X Conjugate.
11. Wash wells 3 times with 1X Wash Buffer making sure each well is filled completely.
12. **Flood entire plate with dH_2O**. Remove contents of wells by inverting over sink and tapping on paper towels.

13. Add 100 µL of Substrate to each well and incubate **in the dark** at room temperature for 30 minutes.
14. Add 100 µL of Stop Solution to each well **in the same order** as the previously added Substrate Solution.
15. Measure absorbance in each well using a spectrophotometric plate reader. It is preferable to read at dual wavelengths of 450/550 nm (or 450/595 nm). A single wavelength of 450 nm can also be used. Wells must be read within 30 minutes of adding the Stop Solution.

Summary of Procedure

Not to be used in place of detailed Assay Procedure. For complete instructions, see above.

	Steps	**Incubation**
1.	Equilibrate kit to room temperature.	-
2.	Add samples and calibrators to wells. Add detector antibody to all wells.	2 hours - room temperature
3.	Wash x3.	-
4.	Add conjugate to all wells.	30 minutes - room temperature
5.	Wash x3.	-
6.	Add substrate to all wells.	30 minutes - room temperature
7.	Add stop solution to all wells.	-
8.	Read plate at 450 nm/550 nm.	-

STORAGE

Store at 4℃.

ANALYSIS OF RESULTS

1. Average the duplicate absorbance values for each calibrator, including the zero, and all sample values.

2. On graph paper, plot the mean absorbance values for each of the calibrators on the Y axis, versus the concentration of each calibrator (ng/mL) on the X axis.

3. Determine the concentration of unknowns by interpolation from the calibration curve. There are a variety of microtiter plate reader software packages available for analysis of microtiter plate data, which simplifies this process.

4. For samples which have been diluted, the PIG3 concentration must be multiplied by the dilution factor (ie., if the sample was diluted five-fold, then the PIG3 value obtained from the calibration curve must be multiplied by five).

PERFORMANCE CHARACTERISTICS

Sensitivity

The lower limit of detection (LLD), commonly used to define sensitivity (Figure 1), was measured by assaying four replicates of zero eight times using two different lots of plates and two different lots of detector antibody. The grand mean signal and pooled standard deviation of zero was calculated. The grand mean of each calibrator (run in replicates of four in eight assays) was used for the calibration curve (Figure 2), and the response, mean signal of zero plus two standard deviations, read in dose from the calibration curve is the LLD; that is, the smallest dose that is not zero that may be determined with 95% confidence.

Figure 1: The assay can easily distinguish 40 pg/mL of PIG3 from zero.

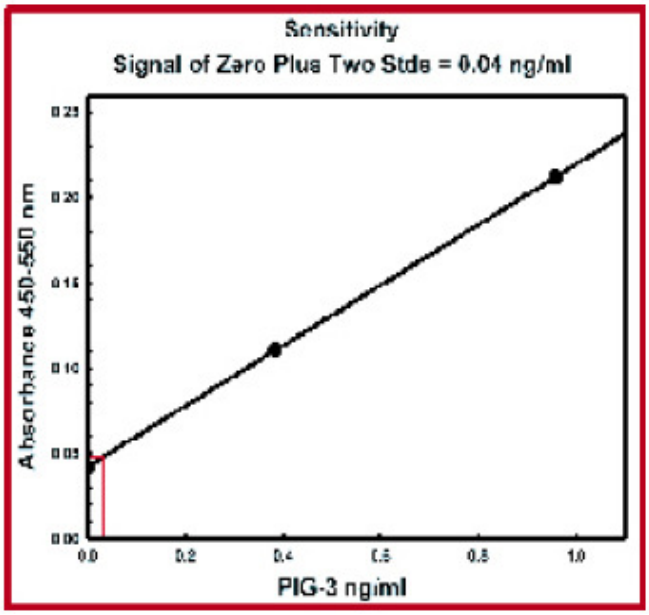
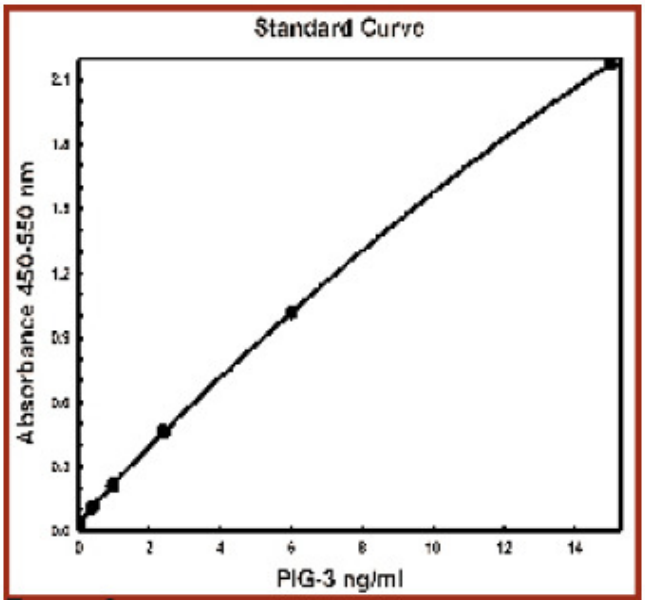


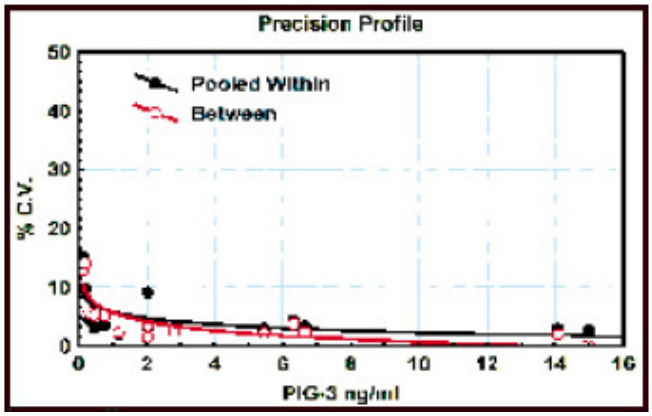
Figure 2: The mean signal of each calibrator run in replicates of four in eight assays using two different lots of plates and two different lots of detector antibody.



Precision

The pooled coefficients of variation and between assay coefficients of variation are plotted against PIG3 levels (Figure 3). The pooled data were collected from samples run eight times using two different lots of plates and two different lots of detector antibody in replicates of four on two separate occasions.

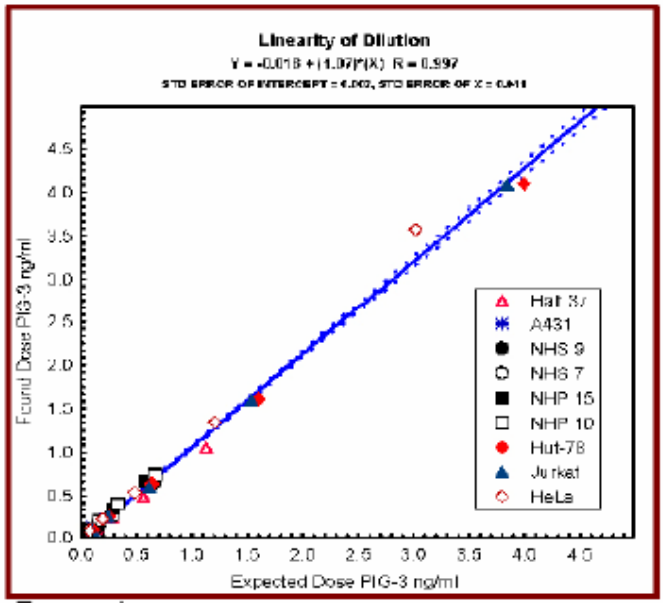
Figure 3: The pooled coefficients of variation and between assay coefficients of variation are plotted against PIG3 level.



Biological Detection Experiments
The PIG3 ELISA detects human PIG3 in sera, plasma, cell culture supernatants and cell lysates. Western blot analysis or direct ELISA data shows the coating and detecting antibodies to be specific for human PIG3.

Linearity
To assess the linearity of the assay, nine samples containing endogenous levels of PIG3 or spiked with various concentrations of human PIG3 were diluted with Sample Diluent and then assayed. The measured human PIG3 concentrations at each dilution within the working range of the assay are within 5% of the expected values for all sample types; sera, plasma, cell supernatants and cell lysates. Results from typical sample dilutions are shown in Figure 4. **Note: serum and plasma samples must be diluted at least 1:4 with Sample Diluent provided.**

Figure 4: The measured human PIG3 concentrations at each dilution within the working range of the assay are within 5% of the expected values for all sample types; sera, plasma, cell supernatants and cell lysates.



Reagent Stability
All of the reagents included with the PIG3 ELISA have been tested for stability. Reagents should not be used beyond the stated expiration date.

FOR RESEARCH USE ONLY

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