29-November-2019

P53 ELISA Protocol AB-000126

For the determination of p53 protein from human tissue, cell lines and other samples.

Please read this protocol completely prior to performing the assay.

Assay Principle

The p53 ELISA kit is designed to quantitatively measure p53 from human tissues, cells or other types of sample. Tumor protein p53, also known as p53, cellular tumor antigen p53 (UniProt name), phosphoprotein p53, tumor suppressor p53, antigen NY-CO-13, or transformation-related protein 53 (TRP53), is any isoform of a protein encoded by homologous genes in various organisms, such as *TP53* (humans) and *Trp53* (mice). This homolog (originally thought to be, and often spoken of as, a single protein) is crucial in multicellular organisms, where it prevents cancer formation, and thus functions as a tumor suppressor. As such, p53 has been described as "the guardian of the genome" because of its role in conserving stability by preventing genome mutation. Hence *TP53* is classified as a tumor suppressor gene. The name p53 was given in 1979 describing the apparent molecular mass; SDS-PAGE analysis indicates that it is a 53-kilodalton (kDa) protein. However, the actual mass of the full-length p53 protein (p53α) based on the sum of masses of the amino acid residues is only 43.7 kDa. an acidic N-terminus transcription-activation domain (TAD), also known as activation domain 1 (AD1), which activates transcription factors. The N-terminus contains two complementary transcriptional activation domains, with a major one at residues 1–42 and a minor one at residues 55–75, specifically involved in the regulation of several pro-apoptotic genes. The protein can be divided into several functional domains: 1. activation domain 2 (AD2) important for apoptotic activity: residues 43-63. 2. proline rich domain important for the apoptotic activity of p53 by nuclear exportation via MAPK: residues 64-92. 3. central DNA-binding core domain (DBD). Contains one zinc atom and several arginine amino acids: residues 102-292. This region is responsible for binding the p53 co-repressor LMO3. 4. Nuclear Localization Signaling (NLS) domain, residues 316-325. homo-oligomerisation domain (OD): residues 307-355. Tetramerization is essential for the activity of p53 *in vivo*. 5. C-terminal involved in downregulation of DNA binding of the central domain: residues 356-393. Mutations that deactivate p53 in cancer usually occur in the DBD. Most of these mutations destroy the ability of the protein to bind to its target DNA sequences, and thus prevents transcriptional activation of these genes. As such, mutations in the DBD are recessive loss-of-function mutations. Molecules of p53 with mutations in the OD dimerise with wild-type p53,

and prevent them from activating transcription. Therefore, OD mutations have a dominant negative effect on the function of p53. Wild-type p53 is a labile protein, comprising folded and unstructured regions that function in a synergistic manner

A series of p53 standards is prepared to generate a standard curve for the assay and all unknown sample concentrations should be read off this standard curve. p53 standards or diluted unknown samples (suggested starting sample concentration for testing 100 µg/ml) are pipetted into the provided 96-well plate which has been pre-coated with polyclonal anti-p53 antibodies (Capturing antibody) to capture p53 from testing samples. Following an incubation to allow capture of the p53 by the antibodies on the plate, a second biotinylated polyclonal anti- p53 antibody (Reporting antibody targeting a different p53 epitope), conjugated with biotin, is added and incubated to allow it to bind to the captured p53s. After 45 min. incubation, the plate is washed and a Streptavidin-HRP (Horse Radish Peroxidase) conjugate is added and incubated for 30 minutes. The Streptavidin-HRP conjugate will be captured by any biotin labeled antibody bound to the plate. Following a wash step to remove unbound conjugate, TMB substrate is added and is converted by the captured HRP to a colored product in proportion to the amount of p53 bound to the plate. After a short incubation to allow color development, the reaction is stopped and the intensity of the generated color is detected in a spectrophotometer plate reader capable of measuring 450nm wavelength. A standard curve will be generated from the p53 standards and used to calculate the concentration of p53 in the unknown samples, after making suitable correction for the dilution of the sample.

Materials and Samples Preparation

PBS
Phosphate-buffered saline, pH 7.4.

PBS-T
Phosphate-buffer saline with 0.1% Tween-20.

Blocking Buffer
Provided as 2X solution.

Coating Plate
A clear plastic microtiter plate is needed to coat with polyclonal (Capturing) anti- p53 IgG. Recommend to use Corning microplate with high binding capability.

P53 Protein Standards
P53 protein stock at 450 ng/ml is provided in 150 µL, referring to the Assay Protocol section for details of standard preparation.

Reporting antibody
A biotin labeled polyclonal antibody is provided with 150 µL/vial. Right before use, do a short spin down to collect the solution, take 100 µL and dilute into 12 ml of blocking buffer to make a working solution.

Streptavidin-HRP Conjugate
A 0.4 mL Streptavidin – Horse Radish Peroxidase conjugate will be used. Immediately prior to the assay, take 0.3 mL of the HRP conjugate and dilute into 12 mL of blocking solution.

TMB Substrate
Use directly without dilution.

Stop Solution
A 1M solution of sulfuric acid. CAUSTIC. Use directly without dilution.

Plate Sealer
One.

Other Materials Required

Distilled or deionized water.
Single- and multi-channel micro-pipettes with disposable tips to accurately dispense volumes 5-250 µL.
Plastic tubes (i.e. 1.5 ml) for sample dilution
Reagent reservoirs for sample addition
Colorimetric 96 well microplate reader capable of reading optical density at 450 nm.
Software for converting raw relative optical density readings from the plate reader and carrying out four parameter logistic curve (4-parameter) fitting. Contact your plate reader manufacturer for details.

Precautions

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete protocol should be read and understood before attempting to use the product.

This kit utilizes a peroxidase-based readout system. Buffers, including other manufacturers Wash Buffers, containing sodium azide will inhibit color production from the enzyme. Make sure **all** buffers used for samples are **azide free**.

The Stop Solution is acid. The solution should not come in contact with skin or eyes. Take appropriate precautions when handling this reagent.

Procedural Notes

Allow diluted reagents and buffers to reach room temperature (18-25°C) prior to starting the assay. Once the assay has been started, all steps should be completed in sequence and without interruption. You do not want the plate to dry out in between steps as this can cause high backgrounds or erroneous results. Make sure that required reagents and buffers are ready when needed. Prior to adding to the plate, reagents should be mixed gently (not vortexed) by swirling.

Avoid contamination of reagents, pipette tips and wells. Use new disposable tips and reservoirs, do not return unused reagent to the stock bottles/vials and do not mix caps of stock solutions.

Incubation time can affect results. All wells should be handled in the same order for each step.

Microplate washing is important and can affect results by giving erroneous results or high backgrounds. We recommend a multichannel pipette or use wash station to add 250 µl of buffer to each well across the plate, followed by a dumping out of contents (to a sink or other receptacle) with a rapid wrist motion. The plate should then be tapped firmly on a paper towel

to shake out any remaining liquid. Avoid prolonged incubation is wash buffer when performing wash steps.

When making additions to the plate, be careful to avoid damaging the coating, for example by scratching the bottoms or the sides of the wells. One technique to avoid this is to make additions (for a right-handed person) from left to right across the plate, supporting the pipette tips on the right edge of the well with each addition and thus avoiding contact with the bottom or sides of the wells.

During the incubation times, the plate should be covered to minimize evaporation from the wells. This can be done with the adhesive covers provided or by stacking an empty plate on top.

After the last wash step and prior to adding the TMB substrate, wipe the bottom of the plate with a clean paper towel to ensure that moisture or fingerprints do not interfere with the OD reading.

Once the TMB substrate is added it will be converted by the captured HRP to a blue colored product. Generally we find that a 15 minutes incubation is sufficient for enough color development to discern differences between the standards and the reaction should be stopped at this point. Bear in mind that, given sufficient time, even a small amount HRP is capable of converting all the TMB to product and if this happens it will be difficult to discern differences between differing concentrations of LON. Keeping OD_{450} values well below 2.0 will result in greatest accuracy as at high absorbance values very little light is reaching the detector and measurements are error prone. (Remember that at an OD of 1.0 only 10% of the light is being detected and at an OD of 2.0 only 1% of the light is reaching the detector).

Assay Protocol

1. Take out 8 siliconized Eppendorf centrifugation tube (1.7 ml), mark the tubes as 90 ng/ml, 30 ng/ml, 10 ng/ml, 3.3 ng/ml, 1.1 ng/ml, 0.37 ng/ml , 0.12 ng/ml and 0 ng/ml respectively. Add 500 µl of blocking solution to the vial marked 90 ng/ml, add 400 µl of blocking buffer to each of the rest vials. P53 protein stock at 450 ng/ml is provided in 150 µL. After a short spin down to collect the solution in the vial, take 100 µl of standard stock and add to 500 µl of blocking buffer in the tube marked 90 ng/ml, mix by inverting the vial a few times. Take 200 µl dilute to the vial marked 30 ng/ml for a 1:3 dilution, repeat the process to dilute down to the 0.12 ng/ml vial. Take 200 µl from the 1 ng/ml and discard. Now all the vials should have 400 µl solution.

2. Use the plate layout sheet on the back page to plan sample layout on plate and also aid in proper sample and standard identification after the assay. We recommend that assays are carried out in duplicate or (preferably) triplicate in order to minimize spurious results.

3. Dilute your sample in 1x blocking buffer to make it 100 µg/mL (this can be adjusted based on the p53 expression level). Make 400 µL for each testing sample.

4. Add both the p53 standards and testing samples based on the plate layout, incubate for 60 min, at room temperature.

5. During the above incubation, take 100 µl of reporting antibody and dilute the reporting antibody to 12 ml of 1x blocking Buffer.

6. Wash plate by emptying contents and adding 250 µL of wash buffer to each well. Empty wells again and tap the plate firmly upside down on a paper towel to fully empty well. Repeat twice.

7. Pipette 100 µL of Reporting Antibody into each well. Cover plate and incubate plate 45 min. at room temperature.

8. During the above incubation, dilute the Streptavidin-HRP conjugate to 0.1 µg/ml in 12 ml of 1x Blocking Buffer.

9. Wash plate by emptying contents and adding 250 µL of wash buffer to each well. Empty wells again and tap the plate firmly upside down on a paper towel to fully empty well. Repeat twice.

10. Pipette 100 µL of Streptavidin-HRP conjugate into wells. Cover plate and incubate plate 30 min. at room temperature.

11. Wash plate by emptying contents and adding 250 µL of wash buffer to each well. Empty wells again and tap the plate firmly upside down on a paper towel to fully empty well. Repeat twice.

12. Add 100 µL of TMB substrate to each well. Monitor color development and stop reaction by adding 100 µL of Stop Solution to each well when color development within standards is sufficient. Generally 15 minutes time will be sufficient.

13. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.

14. Either graph the results on log graph paper or use the plate reader's built-in 4-parameter fit software capabilities to calculate p53 concentration for each sample.

Calculation of Results

Average the triplicate OD readings for each standard, sample and background wells to give a mean OD reading. Subtract the averaged background values from the mean OD values to give a net OD value and create a standard curve using either log graph paper of 4-parameter fit software. Match OD values for the unknowns to [HCP] using the standard curve, remembering to correct for dilution.

Typical data

Sample	Mean OD	[P53]
0.11 ng/ml P53	0.026	
0.34 ng/ml P53	0.081	
1.1 ng/ml P53	0.225	
3.3 ng/ml P53	0.504	
10 ng/ml P53	0.889	
30 ng/ml P53	1.208	
90 ng/ml P53	1.403	
Unknown 2		
Unknown 3		
Unknown 4		

Typical Standard Curve

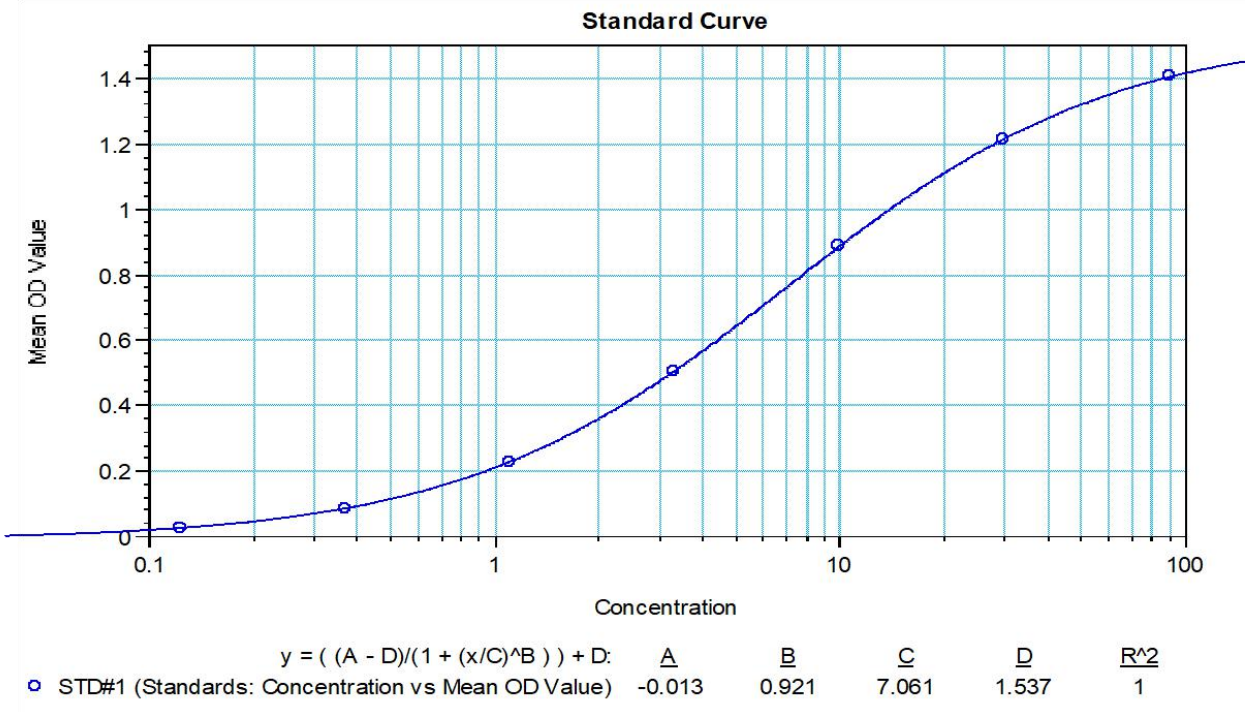

Plate Template

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