Human p53 ELISA Kit

Instructions for use

Catalogue numbers:

1x48 tests: EA101181
1x96 tests: EA101182
2x96 tests: EA101183

For research use only

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Human p53 ELISA KIT

1. Intended use

The OriGeneHuman p53 ELISA is to be used for the in-vitro quantitative determination of p53 in serum, plasma, or cell lysates. The assay will recognize both natural and recombinant p53. The kit recognizes p53 to different extents depending on the source of protein.

This kit has been configured for research use only.

2. Principle of the method

The p53 Kit is a solid phase sandwich Enzyme Linked-Immuno- Sorbent Assay (ELISA). A monoclonal antibody specific for p53 has been coated onto the wells of the microtiter strips provided. Samples, including standards of known p53 concentrations and unknowns are pipetted into these wells.
During the first incubation, the p53 antigen is added to wells. After washing, a biotinylated monoclonal antibody specific for p53 is incubated. Then the enzyme (streptavidin-peroxydase) is added. After incubation and washing to remove all unbound enzyme, a substrate solution which acts on the bound enzyme is added to induce a coloured reaction product. The intensity of this coloured product is directly proportional to the concentration of p53 present in the samples.

3. Reagents provided and reconstitution

Reagents (Store@2-8^{o}C)	**Quantity 1x48 well kit** Cat no. EA101181	**Quantity 1x96 well kit** Cat no. EA101182	**Quantity 2x96 well kit** Cat no. EA101183	**Reconstitution**
96 well microtitrer strip plate	1/2	1	2	Ready to use (Pre-coated)
Plastic plate covers	2	2	4	n/a
Standard: 100 U/ml	1	2	4	Reconstitute as directed on the vial (see Assay preparation, section 8)
Control	1	2	4	Reconstitute as directed on the vial (see Assay preparation, section 8)
Standard Diluent (Buffer)	1 (25ml)	1 (25ml)	1 (25ml)	10x Concentrate, dilute in distilled water (see reagent preparation, section 8)
Standard Diluent (Serum)	1 (7ml)	1 (7ml)	2 (7ml)	Ready to use
Biotinylated anti-p53	1 (0.4ml)	1 (0.4ml)	2 (0.4ml)	Dilute in Biotinylated Antibody Diluent (see Assay preparation, section 8)
Biotinylated Antibody Diluent	1 (7ml)	1 (7ml)	1 (13ml)	Ready to use
Streptavidin-HRP	1 (5µl)	2 (5µl)	4 (5µl)	Add 0.5ml of HRP diluent prior to use (see Assay preparation, section 8)
HRP Diluent	1 (23ml)	1 (23ml)	1 (23ml)	Ready to use
Wash Buffer	1 (10ml)	1 (10ml)	2 (10ml)	200x Concentrate dilute in distilled water (see Assay preparation, section 8)
TMB Substrate	1 (11ml)	1 (11ml)	1 (24ml)	Ready to use
H_2SO_4 stop reagent	1 (11ml)	1 (11ml)	2 (11ml)	Ready to use

4. Materials required but not provided

- Microtiter plate reader fitted with appropriate filters (450nm required with optional 630nm reference filter)
- Microplate washer or wash bottle
- 10, 50, 100, 200 and 1,000µl adjustable single channel micropipettes with disposable tips
- 50-300µl multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Miscellaneous laboratory plastic and/or glass, if possible sterile

5. Storage Instructions

Store kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2-8°C). Expiry of the kit and reagents is stated on box front labels. The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

Wash Buffer: Once prepared store at 2-8° C for up to 1 week
Standard Diluent Buffer: Once prepared store at 2-8° C for up to 1 week
Standards /Controls: Once prepared use immediately and do not store
Biotinylated Secondary Antibody: Once prepared use immediately and do not store
Streptavidin-HRP: Once prepared use immediately and do not store

6. Specimen collection, processing & storage

Cell culture supernatants, serum, plasma or other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

Cell culture supernatants: Remove particulates and aggregates by spinning at approximately 1000 x g for 10 min.

Storage: If not analyzed shortly after collection, samples should be aliquoted (250-500µl) to avoid repeated freeze-thaw cycles and stored frozen at –70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

Recommendation: Do not thaw by heating at 37°C or 56°C. Thaw at room temperature and make sure that sample is completely thawed and homogeneous before use. When possible avoid use of badly haemolysed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

7. Safety & precautions for use

- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures, e.g.CDC/NIH Health manual : " Biosafety in Microbiological and Biomedical Laboratories" 1984.
- Laboratory gloves should be worn at all times.
- Avoid any skin contact with H_2SO_4 and TMB. In case of contact, wash thoroughly with water.
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used.
- Do not pipette by mouth.
- When not in use, kit components should be stored refrigerated or frozen as indicated on vials or bottles labels.
- All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use.
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration.
- Cover or cap all reagents when not in use.
- Do not mix or interchange reagents between different lots.
- Do not use reagents beyond the expiration date of the kit.
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of H_2SO_4 and substrate solution, avoid pipettes with metal parts.
- Use a clean plastic container to prepare the washing solution.
- Thoroughly mix the reagents and samples before use by agitation or swirling.
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells.
- The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning TMB is toxic avoid direct contact with hands. Dispose of properly.
- If a dark blue colour develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded. Read absorbance’s within 1 hour after completion of the assay.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.
- Follow incubation times described in the assay procedure.
- Dispense the TMB solution within 15 min of the washing of the microtiter plate.

8. Assay Preparation

Bring all reagents to room temperature before use

8.1. Assay Design

Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard and zero should be tested **in duplicate**. Remove sufficient Microwell Strips for testing from the pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8^{o}C storage.

Example plate layout(example shown for a 6 point standard curve)

	Standards U/ml		Sample Wells									
	1	2	3	4	5	6	7	8	9	10	11	12
A	100	100										
B	50	50										
C	25	25										
D	12.5	12.5										
E	6.25	6.25										
F	3.12	3.12										
G	Blank	Blank										
H	CTRL	CTRL										

All remaining empty wells can be used to test samples in duplicate

8.2. Preparation of Wash Buffer

Dilute the (200x) wash buffer concentrate 200 fold with distilled water to give a 1x working solution. Pour entire contents (10 ml) of the Washing Buffer Concentrate into a clean 2,000 ml graduated cylinder.Bring final volume to 2,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. Transfer to a clean wash bottle and store at 2˚-8˚C for up to 1 week.

8.3. Preparation of Standard Diluent Buffer

Add the contents of the vial (10x concentrate) to 225ml of distilled water before use.

This solution can be stored at 2-8^{o}C for up to 1 week.

8.4. Preparation of Standard

Depending on the type of samples you are assaying, the kit includes two standard diluents. Because biological fluids might contain proteases or cytokine-binding proteins that could modify the recognition of the cytokine you want to measure. You should reconstitute standard vials with the most appropriate Standard Diluent. For serum and plasma samples use Standard Diluent (Serum) and for cells culture supernatants use Standard diluent (Buffer).
Standard vials must be reconstituted with the volume of Standard Diluent shown on the vial immediately prior to use.This reconstitution gives a stock solution of 100 U/ml of p53. **Mix the reconstituted standard gently by inversion only.**Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 100 to 3.12 U/ml. A fresh standard curve should be produced for each new assay.

- Immediately after reconstitution add 200µl of the reconstituted standard to wells A1 and A2, which provides the highest concentration standard at 100 U/ml
- Add 100µl of appropriate Standard Diluent to the remaining standard wells B1 and B2 to F1 and F2
- Transfer 100µl from wells A1 and A2 to B1 and B2. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells
- Continue this 1:1 dilution using 100µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 100 U/ml to 3.12 U/ml
- Discard 100µl from the final wells of the standard curve (F1 and F2)

Alternatively these dilutions can be performed in separate clean tubes and immediately transferred directly into the relevant wells.

8.5. Preparation of Controls

Freeze-dried control vials should also be reconstituted with the most appropriate diluent to your samples. For serum and plasma samples use standard diluent (Serum) and for cells culture supernatants use Standard diluent (Buffer). Control vials have to be reconstituted with the volume of standard buffer diluent indicated on the vial. Reconstitution of the freeze-dried material with the indicated volume, will give a solution for which the p53 concentration is stated on the vial. Allow control to stand for 5 minutes with gentle swirling prior to distribute in control wells. Do not store after use.

8.6. Preparation of Biotinylated anti-p53

It is recommended this reagent is prepared immediately before use. Dilute the biotinylated anti-p53 with the biotinylated antibody diluent in an appropriate clean glass vial using volumes appropriate to the number of required wells. Please see example volumes below:

Number of wells required	Biotinylated Antibody (µl)	Biotinylated Antibody Diluent (µl)
16	40	1060
24	60	1590
32	80	2120
48	120	3180
96	240	6360

8.7. Preparation of Streptavidin-HRP

It is recommended to centrifuge vial for a few seconds in a microcentrifuge to collect all the volume at the bottom.

Dilute the 5µl vial with 0.5ml of HRP diluent **immediately before use.**Do-not keep this diluted vial for future experiments. Further dilute the HRP solution to volumes appropriate for the number of required wells in a clean glass vial. Please see example volumes below:

Number of wells required	Streptavidin-HRP (µl)	Streptavidin-HRP Diluent (ml)
16	30	2
24	45	3
32	60	4
48	75	5
96	150	10

9. Method

We strongly recommend that every vial is mixed thoroughly without foaming prior to use except the standard vial which must be mixed gently by inversion only.

Prepare all reagents as shown in section 8.
Note: Final preparation of Biotinylated anti-p53 (section 8.6) and Streptavidin-HRP (section 8.7) should occur immediately before use.

Assay Step		Details
1.	Addition	**Prepare Standard curve** as shown in section 8.4
2.	Addition	Add 100 µl of appropriate standard diluent to the blank wells
3.	Addition	Add 100 µl of sample to designated sample wells and add100 µl of the reconstituted control to the control wells
4.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **2hours**
5.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of **1x washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c another two times
6.	Addition	Add 50µl of diluted **biotinylated anti-p53** to all wells
7.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1hour**
8.	Wash	Repeat wash step 5.
9.	Addition	Add 100µl of **Streptavidin-HRP** solution into all wells
10.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **30 min**
11.	Wash	Repeat wash step 5.
12.	Addition	Add 100µl of ready-to-use **TMB Substrate Solution** into all wells
13.	Incubation	Incubate in the dark for **10-20 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil
14.	Addition	Add 100µl of **H_2SO_4:Stop Reagent** into all wells
Read the absorbance value of each well (immediately after step 14.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 630 nm as the reference wave length (610 nm to 650 nm is acceptable).		

***Incubation time of the substrate solution is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range*

10. Data Analysis

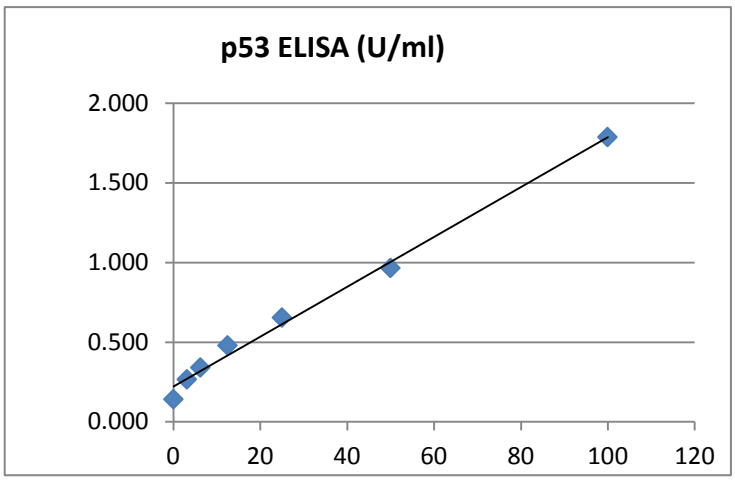
Calculate the average absorbance values for each set of duplicate standards and samples**.** Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding p53 standard concentration on the horizontal axis.

The amount of p53 in each sample is determined by extrapolating OD values against p53 standard concentrations using the standard curve**.**

Examplep53 Standard curve

Standard	p53 Conc.	OD (450nm) mean	CV (%)
1	100	1.788	0.2
2	50	0.965	3.3
3	25	0.654	3.8
4	12.5	0.478	1.6
5	6.25	0.340	0.6
6	3.12	0.266	3.7
Zero	0	0.123	-



Note; curve shown above should not be used to determine results. Every laboratory must produce a standard curve for each set of microwell strips assayed.

11. Assay limitations

Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard diluent or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.

The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced...) on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results.The rate of degradation of native p53 in various matrices has not been investigated.

Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Washing Buffer, fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

As with most biological assays conditions may vary from assay to assay therefore **afresh standard curve must be prepared and run for every assay.**

12. Performance Characteristics

12.1. Sensitivity

The minimum detectable dose of p53 is **<1.5 U/ml**. This has been determined by adding 3 standard deviations to the mean optical density obtained when the zero standard was assayed 32 times.

12.2. Precision

Intra-assay

Sample	n	Mean (U/mL)	SD	CV%
A	8	53.6	1.39	2.6 %
B	8	14.7	0.62	4.2 %

Inter-assay

Sample	n	Mean (U/mL)	SD	CV%
A	5	29.1	2.0	6.8
B	4	5.87	0.55	9.3

13. References

El. Far. et al. Evaluation of serum levels of p53 in hepatocellular carcinoma in Egypt. Clin Chem Lab Med., 2006; 44(5): 653-6

14. Assay Summary

Total procedure length : 3h45mn

Add 100µl of sample or diluted standardor control
↓
Incubate 2 hours at room temperature
↓
Wash three times
↓
Add 50µl Biotinylated anti-p53
↓
Incubate 1 hour at room temperature
↓
Wash three times
↓
Add 100µl of Streptavidin-HRP
↓
Incubate 30min at room temperature
↓
Wash three times
↓
Add 100 µl of ready-to-use TMB
Protect from light. Let the colour develop for 10-20 min.
↓
Add 100µl of H_2SO_4
↓
Read Absorbance at 450 nm

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