

Instructions for Use

IBL

p53 ELISA

Enzyme immunoassay for the quantitative determination of p53 in cell culture supernatant, human serum and plasma

REF **BE59261**

 **96**

   **2-8°C**

EU: *For research
use only.*

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1. INTENDED USE

The p53 ELISA is an enzyme-linked immunosorbent assay for quantitative detection of human p53 in cell culture supernatants, human serum, plasma, or other body fluids. **The p53 ELISA is for research use only. Not for use in diagnostic or therapeutic procedures.**

2. SUMMARY

p53 is the most commonly mutated gene in human cancer. Mutations and allele loss in the gene located on chromosome 17p are the most frequent alterations yet identified in human malignancies. The p53 protein is highly conserved the evolution and expressed in most normal tissues. Wild-type p53 has been shown to be a sequence-specific transcription factor, directly interacting with various cellular and viral protein (10,11).

p53 is considered a stress response gene, the p53 protein acts to induce cell cycle arrest or apoptosis in response to DNA damage, thereby maintaining genetic stability in the organism. These functions are executed by a complex and incompletely understood series of steps known as the p53 pathway. (18)

Initially described as an oncogene, p53 was shown to be capable of suppressing the proliferation of transformed cells. Studies furthermore demonstrated that intact p53 function is essential for the maintenance of the non-tumorigenic phenotype of cells. (17)

Thus p53 plays a vital role in suppressing the development of cancer. (9,12)

p53 is a tumor suppressor gene which induces apoptosis. An inverse relationship in some neoplasms has been shown between p53 and Bcl-2, a proto-oncogene inhibiting apoptosis. (2)

The Bcl-2 homologue BAX gene has also been characterized to be regulated by p53; BAX acts to accelerate the rate at which apoptosis proceeds. (16)

p53 was further shown to mediate apoptosis through cell surface trafficking of APO-1/Fas. (1)

p53 as a transcription factor induces the expression of p21WAF1/CIP1/Sdi1 leading to G1 arrest of the cell. p53 is known to be regulated by phosphorylation by a number of specific protein kinases. Activation by autoproteolysis has been shown. (13)

In addition to proliferation control, a role for p53 in cell senescence has been described. (3)

More than 500 mutations in the p53 gene have been described. (15). These mutations were found in various types of malignancies, hematologic as well as solid tumors. However, all the mutants are not necessarily equivalent in terms of biological activity. (19)

The mutational spectrum differs among cancers of the colon (14), lung, esophagus (8), breast (7,21), liver brain, skin (4), and hemopoietic tissues (5,6).

The chemotherapy-supported p53 gene therapy is under clinical investigation (20).

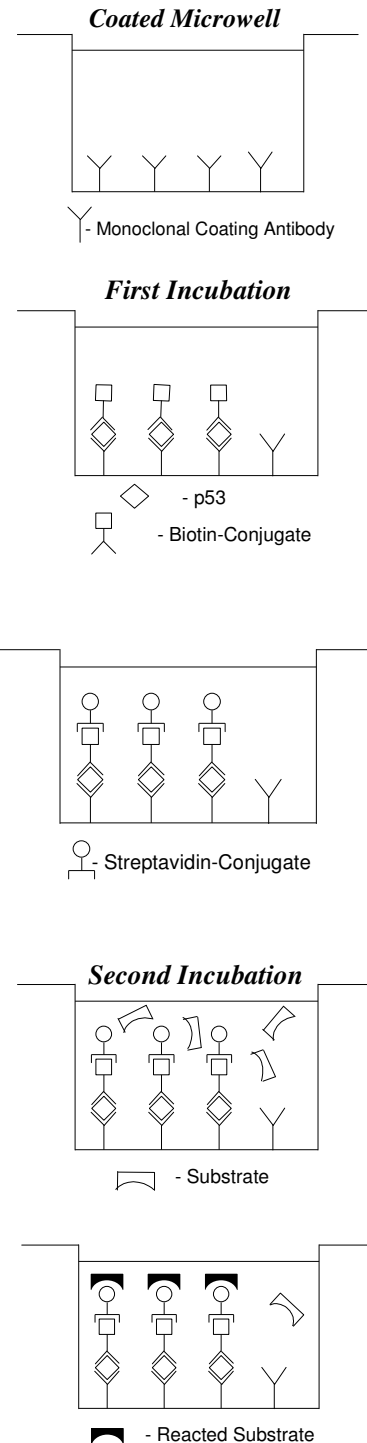
3. PRINCIPLES OF THE TEST

An anti-p53 monoclonal coating antibody is adsorbed onto microwells.

p53 present in the sample or standard binds to antibodies adsorbed to the microwells; a biotin-conjugated monoclonal anti-p53 antibody is added and binds to p53 captured by the first antibody.

Following incubation unbound biotin-conjugated anti-p53 is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-p53. Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

A coloured product is formed in proportion to the amount of p53 present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from seven p53 standard dilutions and p53 sample concentration determined.



4. REAGENTS PROVIDED

- 1 aluminium pouch with a **Microwell Plate coated with Monoclonal Antibody** (murine) to human p53
- 1 vial (100 μ l) **Biotin-Conjugate** anti-p53 monoclonal (murine) antibody*
- 2 vials **p53 Standard**, lyophilized, 100 U/ml upon reconstitution
- 1 vial (150 μ l) **Streptavidin-HRP***
- 1 bottle (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)*
- 1 vial (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20 and 10% BSA)*
- 1 vial (12 ml) **Sample Diluent** (protein matrix)
(Please note: In some cases the Sample Diluent contains insoluble white precipitations which do not interfere with the test performance. Use according to protocol.)
- 1 vial (7 ml) **Substrate Solution I** (tetramethyl-benzidine)
- 1 vial (7 ml) **Substrate Solution II** (0.02 % buffered hydrogen peroxide)
- 1 vial (12 ml) **Stop Solution** (1M Phosphoric acid)
- 1 vial (0.4 ml) **Blue-Dye**
- 1 vial (0.4 ml) **Green-Dye**
- 1 vial (0.4 ml) **Red-Dye**
- 4 adhesive **Plate Covers**
Reagent Labels

* reagents contain preservative

5. STORAGE INSTRUCTIONS

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

6. SPECIMEN COLLECTION

Cell culture supernatants, human 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.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Clinical samples should be kept at 2° to 8°C and separated rapidly before storing at -20°C to avoid loss of bioactive P53. If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles.

For stability and suitability of samples refer to 13. E, and F.

7. MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes
- 10 μ l to 1,000 μ l adjustable single channel micropipettes with disposable tips
- 50 μ l to 300 μ l adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform linear regression analysis.

8. PRECAUTIONS FOR USE

- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Reagents containing thimerosal as preservative may be toxic if ingested.
- Avoid contact of substrate solutions with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.

- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagents.
- Exposure to acids will inactivate the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solutions must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0 % sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

9. PREPARATION OF REAGENTS

Prepare Wash Buffer (reagent A) and Assay Buffer (reagent B) before starting with the test procedure.

A. Wash Buffer

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** into a clean 1,000 ml graduated cylinder. Bring final volume to 1,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that the Wash Buffer is stable for 30 days. Wash Buffer may be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

B. Assay Buffer

Mix the contents of the bottle well. Add contents of **Assay Buffer Concentrate** (5.0 ml) to 95 ml distilled or deionized water and mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer is stable for 30 days. Assay Buffer may be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

C. Preparation of Biotin-Conjugate

Make a 1:100 dilution of the Biotin-Conjugate with **Assay Buffer** (reagent B) in a clean plastic tube.

The Biotin-Conjugate may be prepared as needed according to the following table:

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

D. Preparation of Streptavidin-HRP

Make a 1:100 dilution of the concentrated Streptavidin-HRP solution as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

E. Preparation of p53 Standard

Reconstitute **Standard** by addition of distilled water. Reconstitution volume is stated on the label of the standard vial. Shake gently to ensure complete solubilisation. Discard reconstituted standard not needed.

F. TMB Substrate Solution

Using clean pipettes and containers known to be metal free, dispense an equal volume of **Substrate Solution I** into **Substrate Solution II** and swirl gently to mix. The TMB Substrate Solution may develop a yellow tinge over time. This does not seem to affect product performance. A blue colour present in the TMB Substrate Solution, however, indicates that it has been contaminated and must be discarded. The TMB Substrate Solution must be used within a few minutes after mixing. Warm to room temperature before use. Avoid direct exposure of TMB reagents to intense light and oxidizing agents during storage or incubation.

Substrate preparation by assay size:

Number of Strips	Substrate Solution I (ml)	Substrate Solution II (ml)
1 - 6	3.0	3.0
1 - 12	6.0	6.0

G. Addition of colour-giving reagents: Blue-Dye, Green-Dye, Red-Dye

This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.

Alternatively, the dye solutions from the stocks provided (**Blue-Dye**, **Green-Dye**, **Red-Dye**) can be added to the reagents according to the following guidelines:

- 1. Diluent:** Before sample dilution add the **Blue-Dye** at a dilution of 1:250 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of **Blue-Dye**, proceed according to the instruction booklet.

5 ml Sample Diluent	20 μ l Blue-Dye
12 ml Sample Diluent	48 μ l Blue-Dye

2. Biotin-Conjugate: Before dilution of the concentrated conjugate, add the **Green-Dye** at a dilution of 1:100 (see table below) to the Assay Buffer used for the final conjugate dilution. Proceed after addition of **Green-Dye** according to the instruction booklet, preparation of Biotin-conjugate.

3 ml Assay Buffer	30 μ l Green-Dye
6 ml Assay Buffer	60 μ l Green-Dye
12 ml Assay Buffer	120 μ l Green-Dye

3. Streptavidin-HRP: Before dilution of the concentrated Streptavidin-HRP; add the **Red-Dye** at a dilution of 1:250 (see table below) to the Assay Buffer used for the final Streptavidin-HRP dilution. Proceed after addition of **Red-Dye** according to the instruction booklet, preparation of Streptavidin-HRP.

6 ml Assay Buffer	24 μ l Red-Dye
12 ml Assay Buffer	48 μ l Red-Dye

10. TEST PROTOCOL

- a. Mix all reagents thoroughly without foaming before use.
- b. Determine the number of Microwell Strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank, and optional control sample should be assayed in duplicate. Remove extra **Microwell Strips coated with Monoclonal Antibody** (murine) to human p53 from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
- c. Wash the microwell strips twice with approximately 300 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells.

After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

- d. Add 100 µl of **Sample Diluent** in duplicate to all standard wells. Prepare standard dilutions by pipetting 100 µl of reconstituted (refer to preparation of reagents, 9.E.) **p53 Standard**, in duplicate, into well A1 and A2 (see Figure 1 and 2). Mix the contents of wells A1 and A2 by repeated aspiration and ejection, and transfer 100 µl to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure five times, creating two rows of p53 standard dilutions ranging from 50 to 0.8 U/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of p53 standard dilutions:

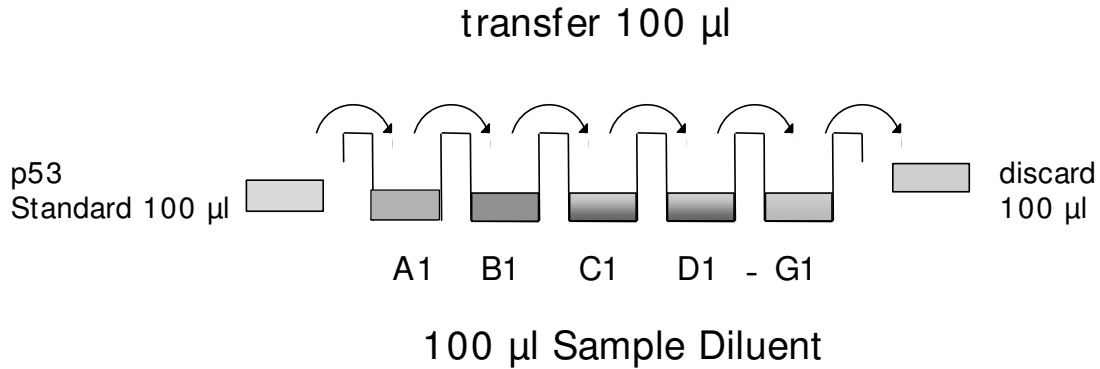


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (50 U/ml)	Standard 1 (50 U/ml)	Sample 1	Sample 1
B	Standard 2 (25 U/ml)	Standard 2 (25 U/ml)	Sample 2	Sample 2
C	Standard 3 (12.5 U/ml)	Standard 3 (12.5 U/ml)	Sample 3	Sample 3
D	Standard 4 (6.3 U/ml)	Standard 4 (6.3 U/ml)	Sample 4	Sample 4
E	Standard 5 (3.2 U/ml)	Standard 5 (3.2 U/ml)	Sample 5	Sample 5
F	Standard 6 (1.6 U/ml)	Standard 6 (1.6 U/ml)	Sample 6	Sample 6
G	Standard 7 (0.8 U/ml)	Standard 7 (0.8 U/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

- e. Add 100 μ l of **Sample Diluent** in duplicate to the blank wells.
- f. Add 50 μ l of **Sample Diluent** to the Sample wells.
- g. Add 50 μ l of each **Sample**, in duplicate, to the designated wells.
- h. Prepare **Biotin-Conjugate** (refer to preparation of reagents 9.C.).
- i. Add 50 μ l of diluted **Biotin-Conjugate** to all wells, including the blank wells.
- j. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 2 hours, if available on a rotator set at 100 rpm.
- k. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.
- l. Prepare Streptavidin-HRP (refer to preparation of reagents D).
- m. Add 100 μ l of diluted **Streptavidin-HRP** to all wells, including the blank wells.
- n. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 1 hour, if available on a rotator set at 100 rpm.
- o. Prepare TMB Substrate Solution a few minutes prior to use (refer to preparation of reagents 9.F.).
- p. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.
- q. Pipette 100 μ l of mixed **TMB Substrate Solution** to all wells, including the blank wells.

- r. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 minutes, if available on a rotator set at 100 rpm. Avoid direct exposure to intense light.

The colour development on the plate should be monitored and the substrate reaction stopped (see point s. of this protocol) before positive wells are no longer properly recordable.

It is recommended to add the stop solution when the highest standard has developed a dark blue colour.

Alternatively the colour development can be monitored by the ELISA reader at 620nm. The substrate reaction should be stopped as soon as an OD of 0.6 – 0.65 is reached.

- s. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8 °C in the dark.
- t. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both, the samples and the sICAM-1 standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

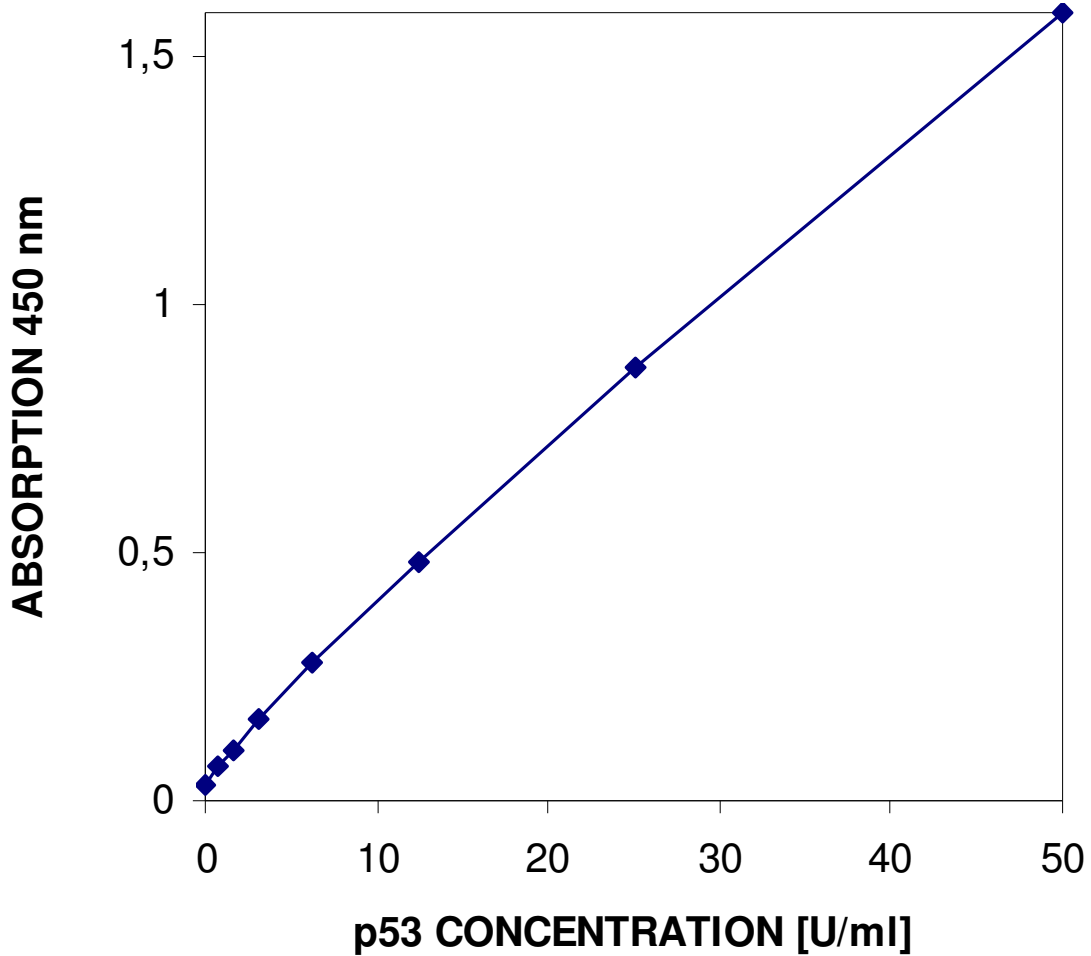
11. CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the p53 concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating p53 for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding p53 concentration.
- **For samples which have been diluted according to the instructions given in this manual 1:2, the concentration has to be multiplied by the dilution factor (x2).**

Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low p53 levels. Such samples require further dilution of 1:4 - 1:8 with Sample Diluent in order to precisely quantitate the actual p53 level.

- It is suggested that each testing facility establishes a control sample of known p53 concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.
- A representative standard curve is shown in Figure 3. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 3. Representative standard curve for p53 ELISA. p53 was diluted in serial two-fold steps in Sample Diluent, symbols represent the mean of three parallel titrations. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.



Typical data using the p53 ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	p53 Concentration (U/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	50	1.589	1.586	0.3
	50	1.583		
2	25	0.889	0.871	2.9
	25	0.853		
3	12.5	0.485	0.479	1.8
	12.5	0.473		
4	6.3	0.279	0.276	1.5
	6.3	0.273		
5	3.2	0.165	0.164	1.3
	3.2	0.162		
6	1.6	0.097	0.099	2.9
	1.6	0.101		
7	0.8	0.066	0.067	2.1
	0.8	0.068		
Blank	0	0.036	0.033	
	0	0.029		

12. LIMITATIONS

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- 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 Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of immunotherapy has significantly increased the number of patients with human anti-mouse IgG antibody (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the Sample Diluent.

13. PERFORMANCE CHARACTERISTICS

A. Sensitivity

The limit of detection of p53 defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (mean plus two standard deviations) was determined to be 0.33 U/ml (mean of 6 independent assays).

B. Reproducibility

a. Intra-assay

Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 7 serum samples containing different concentrations of p53. Two standard curves were run on each plate. Data below show the mean p53 concentration and the coefficient of variation for each sample. The overall intra-assay coefficient of variation has been calculated to be 5.5%.

Positive Sample	Experiment	p53 Concentration (U/ml)	Coefficient of Variation (%)
1	1	4.2	3.9
	2	3.8	6.7
	3	3.9	1.7
2	1	11.0	5.5
	2	11.1	10.3
	3	9.9	2.8
3	1	13.5	7.5
	2	11.5	6.2
	3	11.4	4.8
4	1	3.2	7.6
	2	2.5	4.6
	3	3.1	8.4
5	1	2.1	8.5
	2	2.1	10.3
	3	2.4	11.3
6	1	5.8	1.0
	2	5.0	0.7
	3	4.7	6.1
7	1	16.3	2.4
	2	13.5	1.5
	3	14.4	2.5

b. Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 7 serum samples containing different concentrations of p53. Two standard curves were run on each plate. Data below show the mean p53 concentration and the coefficient of variation calculated on 18 determinations of each sample. The overall inter-assay coefficient of variation has been calculated to be 8.9 %.

Sample	p53 Concentration (U/ml)	Coefficient of Variation (%)
1	4.0	4.8
2	10.7	6.2
3	12.1	9.8
4	2.9	13.9
5	2.2	6.7
6	5.2	10.8
7	14.7	9.9

C. Spike Recovery

The spike recovery was evaluated by spiking two levels of p53 into four normal human sera. Recoveries were determined in two independent experiments with 4 replicates each. The amount of endogenous p53 in unspiked serum was subtracted from the spike values. Recoveries ranged from 80 to 118% with an overall mean recovery of 95%.

D. Dilution Parallelism

Four serum samples with different levels of p53 spiked and unspiked were assayed at four serial two-fold dilutions (1:2-1:16) with 4 replicates each. In the table below the per cent recovery of expected values is listed. Recoveries ranged from 84% to 121% with an overall mean recovery of 102 %.

Sample	Dilution	p53 Concentration (U/ml)		
		Expected Value	Observed Value	% Recovery of Exp. Value
1	1:2	--	14.5	--
	1:4	7.2	8.1	112
	1:8	3.6	3.5	98
	1:16	1.8	1.5	84
2	1:2	--	10.3	--
	1:4	5.2	5.6	109
	1:8	2.6	3.1	121
	1:16	1.3	1.4	108
3	1:2	--	18.2	--
	1:4	9.1	8.2	90
	1:8	4.6	4.3	95
	1:16	2.3	2.6	116
4	1:2	--	11.5	--
	1:4	5.7	5.7	99
	1:8	2.9	2.6	92
	1:16	1.4	1.5	105

E. Sample Stability

a. Freeze-Thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored at -20°C and thawed up to 5 times, and p53 levels determined. There was no significant loss of p53 by freezing and thawing up to 3 times, however values were slightly lower after 5 freeze-thaw cycles.

b. Storage Stability

Aliquots of a serum sample (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the p53 level determined after 24 h. There was no significant loss of p53 immunoreactivity during storage under above conditions.

F. Comparison of Serum and Plasma

From two individuals, serum as well as EDTA and citrate, and heparin plasma obtained at the same time point were evaluated. p53 concentrations were not significantly different and therefore all these body fluids are suitable for the assay. It is nevertheless highly recommended to assure the uniformity of blood preparations.

G. Specificity

The interference of circulating factors of the immune systems was evaluated by spiking these proteins at physiologically relevant concentrations into a serum sample. There was no detectable cross reactivity.

H. Expected Values

A panel of 22 sera from apparently healthy blood donors (males and females) was tested for p53. There were no p53 levels detected in the tested control collective. The normal levels measured may however vary with the sample collective used.

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15. REAGENT PREPARATION SUMMARY

A. Wash Buffer Add **Wash Buffer Concentrate** 20 x (50 ml) to 950 ml distilled water

B. Assay Buffer	Number of Strips	Assay Buffer Concentr. (ml)	Distilled Water (ml)
	1 - 6	2.5	47.5
	1 - 12	5.0	95.0

C. Biotin-Conjugate Make a 1:100 dilution according to the table.

	Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (ml)
	1 - 6	0.03	2.97
	1 - 12	0.06	5.94

D. Streptavidin-HRP	Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (ml)
	1 - 6	0.06	5.94
	1 - 12	0.12	11.88

E. Standard Reconstitute **Standard** by addition of distilled water. Reconstitution volume is stated on the label of the standard vial

F. TMB Substrate Solution	Number of Strips	Substrate Solution I (ml)	Substrate Solution II (ml)
	1 - 6	3.0	3.0
	1 - 12	6.0	6.0

16. TEST PROTOCOL SUMMARY

- Wash microwell strips twice with **Wash Buffer**
- Add 100 µl **Sample Diluent**, in duplicate, to all standard wells.
- Pipette 100 µl reconstituted **p53 Standard** into the first wells and create standard dilutions ranging from 50 to 0.8 U/ml by transferring 100 µl from well to well. Discard 100 µl from the last wells
- Add 100 µl **Sample Diluent**, in duplicate, to the blank wells
- Add 50 µl **Sample Diluent** to sample wells.
- Add 50 µl **Sample**, in duplicate, to designated wells
- Prepare Biotin-Conjugate
- Add 50 µl of diluted **Biotin-Conjugate** to all wells
- Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C)
- Prepare Streptavidin-HRP
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl diluted **Streptavidin-HRP** to all wells
- Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C)
- Prepare TMB Substrate Solution few minutes prior to use
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl of mixed **TMB Substrate Solution** to all wells including blank wells
- Incubate the microwell strips for about 10 to 20 minutes at room temperature (18° to 25°C).
- Add 100 µl **Stop Solution** to all wells including blank wells
- Blank microwell reader and measure colour intensity at 450 nm

Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low p53 levels. Such samples require further dilution of 1:4 - 1:8 with Sample Diluent in order to precisely quantitate the actual p53 level.