CODE No. 7640E

For Research Use Only. Not for use in diagnostic procedures.

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Enzyme immunoassay for the detection of anti-p53 IgG in human serum

MESACUP Anti-p53 TEST

CODE No. 7640E

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Before use, thoroughly read these Instructions.

1. Intended Use

The MESACUP anti-p53 TEST is an Enzyme Linked Immuno-Sorbent assay (ELISA) detection kit for anti-p53 antibody in serum.

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2. Summary and Explanation

The autoantibodies to p53 protein（anti-p53 antibodies）are highly related to malignant diseases and rarely detected in healthy donors and patients with benign diseases[1)]. This immune response is correlated with the presence of a p53 gene mutation which leads to the accumulation of an ineffective p53 protein in the tumor cells[2-5)].

3. Principle

The MESACUP anti-p53 TEST measures anti-p53 antibodies in the serum by ELISA. Calibrators and patient serum are added to microwells coated with recombinant wild-type human p53 protein (to detect specific anti-p53 antibodies) and with control proteins (to detect non-specific interactions), allowing anti-p53 antibodies to react with the immobilized antigen (Sample incubation). After washing to remove any unbound serum proteins, horseradish peroxidase conjugated anti human IgG is added and incubated (Conjugate incubation). Following another washing step, the peroxidase substrate is added and incubated for an additional period of time (Substrate incubation). Acid solution is then added to each well to terminate the enzyme reaction and to stabilize the color development. The value in each sample can be obtained by comparing the OD of the sample to the OD of the anti-p53 standard.

4. Brief assay procedure

<Sample incubation> Add 100 µl of diluted sample (1:101) to each well of microwell plate
(20-30 °C) 60 min. with shaking
↓
Wash
↓
<Conjugate incubation> Add 100 µl of conjugate solution to each well
(20-30°C) 60 min. with shaking
↓
Wash
↓
<Substrate incubation> Add 100 µl of substrate to each well
(20-30 °C) 30 min
↓
Add 100 µl of stop solution to each well
↓
Read absorbance
↓
Interpretation of result

5. Materials provided

Materials	Quantity (48 wells × 2)
p53 Microwell Strips	8-well × 6 strips
Control protein Microwell Strips	8-well × 6 strips
Anti-p53 standard 1- 4 *The concentration is printed to the label.	0.1 mL × 4 conc.
Positive Control	0.1 mL × 1 vial
Negative Control	0.1 mL × 1 vial
Conjugate Reagent (101X) (Peroxidase-conjugated anti-human IgG)	0.2 mL × 1 vial
Conjugate Diluent	24 ml × 1 bottle
Assay Diluent	50 ml × 2 bottles
Wash Concentrate (10 X)	100 ml × 1 bottle
Substrate Solution (TMB/H_2O_2)	20 ml × 1 bottle
Stop Solution	20 ml × 1 bottle
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Microcup in detail:
An 8 well strip on which recombinant p53 protein is coated and an 8 well strip on which Control protein is coated , are set alternately, and 6 strips / microplate is put in one plastic bag (Fig 1). For samples, Blank (Assay Diluent), Anti-p53 standard, Positive Control and Negative Control, test should be done for these two kinds of microcups at the same time.

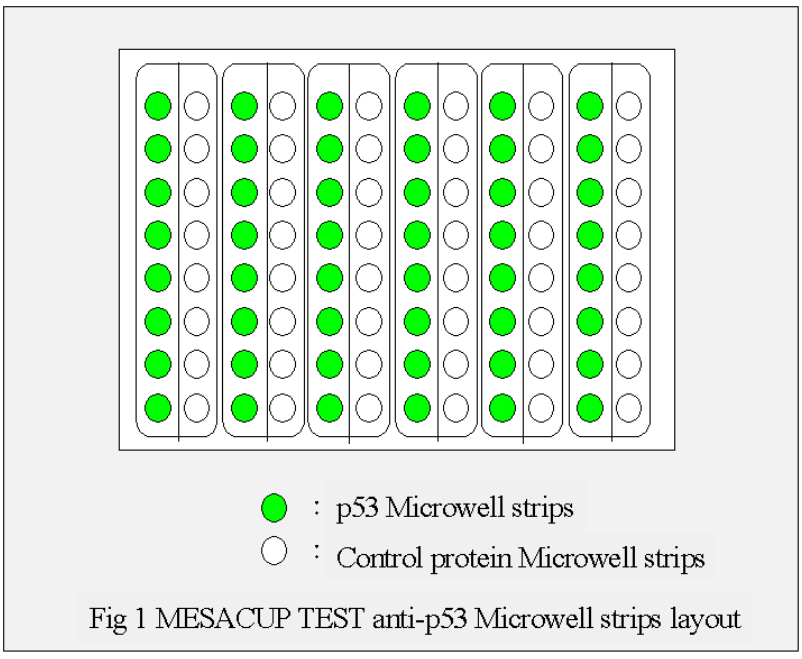

Fig 1 MESACUP TEST anti-p53 Microwell strips layout

6. Materials and equipment required but not provided

- Microplate reader (wavelength: 450 nm, 620 nm/reference)
- Multichannel micropipette (e.g. 100 µl - 300 µl) for dispensing conjugate, substrate, and stop solution.
- Single channel pipette (10 µl & 100 µl)
- Reagent reservoir
- Autowasher or washing bottle
- Deionized or distilled water
- One liter graduated cylinder for preparation of wash solution
- Test tubes for patient sample dilutions (e.g. 1000 µl)
- Disposable pipette tips
- Paper towels
- Micro plate shaker
- Microplate cover

7. Analytical Precautions

- This kit is intended for Research Use Only. Not for use in diagnostic procedures. Not for human, drug, or therapeutic use.
- Do not use kit components beyond the stated expiration dates.
- Avoid contact of reagents with eyes, skin and clothing. Reagents on skin must be washed away with plenty of water. TMB contains irritant and Stop Solution consists of a 0.5 mole/L sulfuric acid, which is poisonous and corrosive.
- Anti-p53 standard 1-4, Positive Control and Negative Control are derived from human serum, in which HBs antigen, HCV antibody, HIV-1 and HIV-2 antibodies have not been detected. No test method, however, can guarantee the absence of these or any other infectious agents. These reagents and all patient samples should be handled as if they are capable of transmitting AIDS, hepatitis or any other infectious diseases.
- Anti-p53 standard 1-4, Positive Control, Negative Control and Assay Diluent contain sodium azide (0.09%) as a preservative and must be handled with caution - do not ingest or allow contact with skin or mucous membranes. Sodium azide may react with copper or lead in plumbing system to form explosive metal azides. Therefore, always flush disposing materials containing sodium azide, with plenty of water into a drain.
- Some kit components contain animal origin materials, which are from non-infectious animals. These components, however, should be treated as potential biohazards in use and for disposal.
- All reagents must be brought to room temperature (20 -30 ^{o}C) before starting the assay.
- Do not expose the kit to direct sun during assay and storage.
- Avoid microbial and cross contamination of reagents or samples.
- Incubation temperatures above or below normal room temperature (20-30 ^{o}C), shorter or longer time periods of incubation and inaccurate dilution may give erroneous results.
- The wells must be rinsed with Wash Solution properly enough to avoid false positive.
- Carefully pipette not to foam each sample and reagent to avoid cross contamination between microwells.
- All microwell strips, which are not immediately required, should be returned to the zip lock pouch, which must be carefully resealed to avoid moisture absorption.
- Wash Concentrate may become turbid at 2-8 ^{o}C, which does not cause inconsistent results.
- Implement used for the test should be disposed or treated as shown below.
- Soak in 2% final conc. glutaraldehyde solution for more than 1 hour or soak in 0.5% Sodium

hypochlorite solution (available chlorine: approx. 5000ppm) for more than 1 hour or autoclave at 121 °C for more than 20 minutes.

8. Procedure

■ PREPARATION OF REAGENTS

1. Bring all assay materials to room temperature (20-30 °C) prior to use.
2. Microwell Strips: Remove required microwell strip from pouch and place them in the frame. Promptly return unused strips to refrigerated storage.
3. Wash Solution: Prepare 1:10 dilution of The Wash Concentrate prior to use. (ex. add 100 ml of Wash Concentrate to 900 ml of distilled water). The diluted wash solution is stable for 2 weeks at 2 -8°C.
4. Dilute the anti-p53 standard 1-4, Positive Control and Negative Control 1:101 by adding 5 µl of serum to 500 µl of Assay Diluent.
5. Dilute Conjugate reagent 1:101 by adding 100 µl of Reagent to 10 ml of Conjugate Diluent.
6. Do not dilute Assay Diluent, Substrate and Stop Solution, which are ready-to-use.

■ PREPARATION OF SAMPLES

1. Use fresh patient sera. Samples are stable for 1 week at 2-8 °C. Aliquot each sample and store below -20°C if necessary. Avoid repeated freezing and thawing.
2. Dilute each patient serum 1:101 by adding 5 µl of serum to 500 µl of Assay Diluent.

*Diluted samples must be used within a day.

* Assay Diluent may form precipitate, which does not cause inconsistent results.

■ ASSAY PROCEDURE

STEP 1. (SAMPLE INCUBATION)

Using the multi-channel pipette, transfer 100 µl of diluted the anti-p53 standard 1-4, Positive Control, Negative Control, Assay Diluent as Blank and each diluted sample into the appropriate microwells of the p53 microwell and the control microwell (cf. Fig 1).

* Incubation starts on pipetting to the antigen-coated microwells. Pipetting should be completed as quickly as possible.

Cover wells with a plate sealer and incubate for 60 minutes at room temperature (20-30 °C) with 250 rpm shaking (micro plate shaker).

STEP 2. (WASHING)

Aspirate or discard the well contents. Fill the well with Wash Solution and then completely aspirate or discard the contents. Wash 6 times. Tap the plate on a paper towel to remove any remaining Wash Solution. When autowasher is used, wash 6 times.

* Each laboratory is recommended to confirm its own appropriate washing times and set- up.

* Wash Solution should be used at 20-30 °C.

STEP 3. (CONJUGATE INCUBATION)

Pour Conjugated Reagent into a reservoir. Add 100 µl of the Conjugated Reagent to each well with multi-channel pipette. Cover wells with the plate sealer and incubate for 60 minutes at room temperature (20-30°C) with 250 rpm shaking (micro plate shaker).

STEP 4. (WASHING)

Wash the microplate following the STEP 2 procedure.

STEP 5. (SUBSTRATE INCUBATION)

Pour Substrate into a reservoir and pipette 100 µl of the Substrate to each well with multi-channel pipette.

*. A new disposable reservoir should be used because Substrate is easily oxidized by metal ions.

* The Substrate, once poured in a reservoir, should not be returned to the bottle.

Cover wells with the plate sealer and incubate for 30 minutes at room temperature **without** shaking (20-30°C).

STEP 6. (STOP REACTION)

Pour Stop Solution into a reservoir. Pipette 100 µl of the solution to each well with multi-channel pipette.

■ READING

Read the absorbance of each well at 450 nm. If a dual wavelength plate reader is available, set the test wavelength at 450 nm and the reference at 620 nm.

* Reading should be done as quickly as possible after stopping the reaction.

* Ensure that the bottom of the plate is clean and dry, and that no air bubbles are present on the surface of the liquid in the wells before reading the plate.

■ RESULTS-INTERPRETATION

Human sera may give rise to variable background signals. For maximum specificity, this protocol uses an internal control to measure the nonspecific background of each sample. This background reflects nonspecific interaction of serum components either with the plastic or with the components used in the ELISA. In the assay, the nonspecific background corresponds to the absorbance measured on wells coated with control proteins.

1. Calculate the average absorbance for each blank, Anti-p53 standard and sample for wells coated with p53 protein.
2. Calculate the average absorbance for each blank, Anti-p53 standard and sample for wells coated with control proteins.
3. Determine the net absorbance by subtracting the assay blank average absorbance from the sample or Anti-p53 standard average absorbance:

   ->sample or Anti-p53 standard (p53 wells) average absorbance - blank (p53 wells) average absorbance

   ->sample or Anti-p53 standard (control proteins wells) average absorbance - blank (control proteins wells) average absorbance
4. Calculate the specific signal:

   [p53 net absorbance] - [control proteins net absorbance]
5. Construct a calibration curve from the specific signals of Anti-p53 standard 1-4 (y axis) and from the levels of antibodies indicated on the anti-p53 standard containing vials (x axis). Levels of anti-p53 antibodies are then determined from the calibration curve. Alternatively a suitable computer and curve-fitting program can be used. If a sample's O.D. is out of range

for the calibration curve, the assay should be repeated with a higher sample dilution with Assay Diluent. The result must be multiplied by the extra-dilution factor.

Interpretation:

Anti-p53 level	Result
> 1.30 U/ml	presence

10. Performance Characteristics

Assay range

Assay range of this kit is 0.70 U/ml – 15.00 U/ml.

Reproducibility

Reproducibility was determined by testing 3 samples (1.00 U/ml-3.00 U/ml, 3.00 U/ml-8.00 U/ml, 8.00 U/ml-15.00 U/ml) for 8 times. CV(%) values for reproducibility and repeatability were below 15% for each sample.

Package size

48 wells×2

Storage and Stability

All kit components must be stored at 2-8°C. All reagents are stable for 12 months after manufacturing when stored at the conditions indicated.

11. References

(1) Crawford, L. V., Int. J. Cancer, 30: 403-408, 1982.

(2) Soussi, T., Cancer Res., 60: 1777-1788, 2000.

(3) Winter, S. F. et al., Cancer Res., 52: 4168-4174, 1992.

(4) von Brevern, M. C. et al., Cancer Res., 56: 4917-4921, 1996.

(5) Iizasa, T. et al., Cancer Immunol. Immunother., 46: 345-349, 1998.