Prepare a protein standard of the target protein. The user will determine sample dilution fold by estimation of target protein content. Before using the kit, quick spin tubes to bring down all solution to the bottom of tube.

Warm diluted ABC and TMB solutions at 37 °C to avoid variable temperature effects. Completely and evenly mix reagents and solutions.

Dilute protein standards by factor of 1:10 in 1x Diluent and 1:100 in 2x Diluent. Prepare a protein standard of the target protein. The user will determine sample dilution fold by estimation of target protein content. Before using the kit, quick spin tubes to bring down all solution to the bottom of tube.

Warm diluted ABC and TMB solutions at 37 °C, place in a 37 °C warm water bath. Warm diluted ABC and TMB solutions at 37 °C, place in a 37 °C warm water bath. Warm diluted ABC and TMB solutions at 37 °C, place in a 37 °C warm water bath.

Washing Buffer (not provided): TBS or PBS

0.01M TBS: Add 1.2g Tris, 8.5g NaCl, 450 μl of purified acetic acid or 700 μl of concentrated hydrochloric acid to 900 ml H2O and adjust pH to 7.2-7.6. Adjust the total volume to 1L.

0.01M PBS: Add 8.5g sodium chloride, 1.4g Na2HPO4, and 0.2g NaH2PO4 to 900ml distilled H2O and adjust pH to 7.2-7.6. Adjust the total volume to 1L.

Storage

Store at 4 °C. Cell Applications, Inc. recommends using the kit within 6 months of order.

BACKGROUNDS

Heat-shock protein 27, Hsp27, (also known as Hsp25 in the mouse), is a member of the small-Hsp family, is found in human cells- both cancer cells and normal cells. In addition to its role in protein folding, Hsp27 intervenes in the modulation of differentiation as well as in apoptosis. Furthermore, it has been identified as a potent regulator of the cytoskeleton as a result of its ability to inhibit actin polymerization. This property is dependent on the phosphorylation state of Hsp27 and its structural organization. Hsp27 can be reversibly phosphorylated on three serine residues in humans (two for Hsp25 in mice) by the mitogen-activated protein kinase-activated kinases 2 and 3 (MK2/3), which are themselves activated by phosphorylation through either the p38 or the extracellular signal-regulated protein kinase (ERK) signaling pathway. Upstream signals, such as differentiating agents and mitogens, that activate MK2/3 also have the ability to induce Hsp27 phosphorylation. In general, it is believed that MK2/3 are the main mediators of Hsp27 phosphorylation, but other kinases such as PKC delta can also phosphorylate Hsp27. This protein, besides its putative role in thermotolerance, is of special clinical interest because of recent data suggesting it may also play a role in drug resistance. In patients with cervical cancer, Hsp27 is predominantly expressed in well-differentiated and moderately differentiated squamous cell carcinomas. In addition, expression of Hsp27 seems to be a negative prognostic factor for gastric cancer. Different isoforms of Hsp27 have been found in lymphoid tissue of patients with acute lympho-blastic leukemia, and the protein has also been associated with viral infections.

ELISA OVERVIEW

Cell Applications ELISA Kits are based on standard sandwich enzyme-linked immunosorbent assay technology. Freshly prepared standards, samples, and solutions are recommended for best results.

1. Prepare test samples.
2. Prepare a protein standard of the target protein.
3. Add test samples and standards to the precoated 96-well plate. Do not wash.
5. Add Avidin-Biotin-Peroxidase Complex (ABC) Solution. Wash.
6. Add Tetramethylbenzidine (TMB) Color Developing Agent, containing HRP substrate.
7. Add TMB Stop Solution
8. Subject the plate to analysis.

NOTES:

• Before using the kit, quick spin tubes to bring down all solution to the bottom of tube.
• Duplicate assay wells are recommended for both standard and sample testing.
• Do not let the 96-well plate dry, this will lead to inactivation of plate components.
• When diluting samples and reagents, ensure that they are mixed completely and evenly.
• Pre-warm diluted ABC and TMB solutions at 37 °C for 30 min before use to avoid variable temperature effects.
• For washes, use TBS or PBS. Do not touch well walls.
• A protein standard is included in the kit. A protein standard detection curve should be generated with each experiment, no more than 2 hours prior to the experiment.
• The user will determine sample dilution fold by estimation of target protein amount in samples.

References

**PROTOCOL**

I. Plate Washing

Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1-2 minutes. Repeat this process two additional times for a total of three washes.

II. Preparation of Test Samples

**Test Sample Processing**

- **Cell culture supernate, tissue lysate or body fluids:** Remove particulates by centrifugation.
- **Serum:** Allow the serum to clot in a serum separator tube (about 2 hours) at room temperature. Centrifuge at approximately 1000 X g for 10 min.

**Sample Dilution Guideline**

Estimate the concentration of the target protein in the sample and select a proper dilution factor such that the diluted target protein concentration falls within the standard curve range. Depending on the sample, several trial dilutions may be necessary. Dilute the sample using the provided diluent buffer, mixing well. Suggested working dilutions of samples are as follows:

<table>
<thead>
<tr>
<th>Target Protein Concentration (ng/ml)</th>
<th>Sample Working Dilution</th>
<th>Sample Vol.</th>
<th>Diluent Buffer Vol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>40-400 ng/ml</td>
<td>1:100</td>
<td>1 μl</td>
<td>99 μl</td>
</tr>
<tr>
<td>4-40 ng/ml</td>
<td>1:10</td>
<td>10 μl</td>
<td>90 μl</td>
</tr>
<tr>
<td>≥62.5 ng/ml</td>
<td>1:2</td>
<td>50 μl</td>
<td>50 μl</td>
</tr>
<tr>
<td>≥62.5 ng/ml</td>
<td>n/a</td>
<td>100 μl</td>
<td>n/a</td>
</tr>
</tbody>
</table>

If samples will be assayed within 24 hours, store at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

III. Preparation of Reagents

**Reconstitution of the Standard**

The standard solutions should be prepared no more than 2 hours prior to the experiment. Two tubes of the standard are included in each kit. Use one tube for each experiment.

1. 10,000 pg/ml of human HSP27 standard solution: Add 1 ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
2. 4000 pg/ml of human HSP27 standard solution: Add 0.4 ml of the above 10 ng/ml HSP27 standard solution into 0.6 ml sample diluent buffer and mix thoroughly.
3. 2000 pg/ml – 62.5 pg/ml of human HSP27 standard solutions: Label 6 Eppendorf tubes with 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, respectively. Aliquot 0.3 ml of the sample diluent buffer into each tube. Add 0.3 ml of the above 4000 pg/ml HSP27 standard solution into 1st tube and mix. Transfer 0.3 ml from 1st tube to 2nd tube and mix. Transfer 0.3 ml from 2nd tube to 3rd tube and mix, and so on.

**Preparation of Biotinylated Antibody Working Solution**

The solution should be prepared no more than 2 hours prior to the experiment.

1. The total volume should be: 0.1 ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
2. Biotinylated antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly.

**Preparation of the Avidin-Biotin-Peroxidase Complex (ABC) Working Solution**

The solution should be prepared no more than 1 hour prior to the experiment.

1. The total volume should be: 0.1 ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
2. Avidin-Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly.

IV. ELISA

The ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 min before use. When diluting samples and reagents, they must be mixed completely and evenly. A standard detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of target protein amount in samples.

1. Aliquot 0.1 ml per well of the 4000 pg/ml, 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml human HSP27 standard solutions into the precoated 96-well plate. Add 0.1 ml of the sample diluent buffer into the control well (Blank well). Add 0.1 ml of each properly diluted sample of human sera, plasma, body fluids, tissue lysates or cell culture supernatants to each empty well. See "Sample Dilution Guideline" above for details. We recommend that each human HSP27 standard solution and each sample is measured in duplicate.
2. Seal the plate with the cover and incubate at 37°C for 90 min.
3. Remove the cover, discard plate content, and blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.
4. Add 0.1 ml of biotinylated anti-human HSP27 antibody working solution into each well and incubate the plate at 37°C for 60 min.
5. Wash plate 3 times with 0.01 M TBS or 0.01 M PBS, and each time let washing buffer stay in the wells for 1 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. (Plate Washing Method: Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1-2 minutes. Repeat this process two additional times for a total of THREE washes. Note: For automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling wells with PBS or TBS buffer. Blot the plate onto paper towels or other absorbent material.)
6. Add 0.1 ml of prepared ABC working solution into each well and incubate the plate at 37°C for 30 min.
7. Wash plate 5 times with 0.01 M TBS or 0.01 M PBS, and each time let washing buffer stay in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. (See Step 5 for plate washing method).
8. Add 90 μl of prepared TMB color developing agent into each well and incubate plate at 37°C in dark for 20-25 min (Note: For reference only, the optimal incubation time should be determined by end user. And the shades of blue can be seen in the wells with the four most concentrated human HSP27 standard solutions; the other wells show no obvious color).
9. Add 0.1 ml of prepared TMB stop solution into each well. The color changes into yellow immediately.
10. Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution.

V. Calculating Protein Concentration

- **For all wells,** determine O.D.450(Reading) – O.D.450(Blank)
- Plot the standard curve: Plot O.D.450(Reading) of each standard solution (Y) vs. the respective concentration of the standard solution (X). See **Figure 1** for a typical standard curve.
- The target protein concentration in samples can be interpolated from the standard curve. Multiply the interpolated concentration by the dilution factor to obtain the target protein concentration in the sample.

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