SIGNIFICANCE AND BACKGROUND

Bullous pemphigoid (BP) is a chronic itchy blistering disorder found mainly in aged persons, characterized by frequent occurring of tense blister and erythema. IgG anti-basement membrane zone (BMZ) antibodies are found in the serum of patients, and linear IgG or C3 sediment is found on the basement membrane zone of the lesion. Target antigens of the autoantibodies in BP patient serum are BP180 and BP230\(^1\), also called BPAG2 and BPAG1. Molecular weight of these antigens is 180 kD and 230 kD respectively.

Anti-BP180 is thought to be the pathogenic autoantibody, however, not all BP patients have anti-BP180 antibody in their serum\(^2\). Anti-BP230 antibody is considered to be a useful serologic marker of the disease\(^3\).

The BP230 ELISA Kit has recombinant protein of both N-terminus and C-terminus of BP230 as solid phase and measures anti-BP230 autoantibodies in patient serum.

INTENDED USE

The BP230 ELISA Kit is a semi-quantitative enzyme-linked immunosorbent assay (ELISA) for the detection of anti-BP230 antibodies in human serum. The BP230 ELISA Kit is intended for in vitro diagnostic use as an aid in the diagnosis of bullous pemphigoid in conjunction with other laboratory and clinical findings. Patients with Bullous Pemphigoid are known to have either BP180 or BP230 or both types of antibodies in serum. It is recommended that each patient be tested for BP180 and BP230 antibodies.

PRINCIPLE OF THE PROCEDURE

The BP230 ELISA Kit measures anti-BP230 antibodies present in the serum by ELISA. Calibrators and patient sera are added to microwell coated with BP230 antigen, allowing anti-BP230 antibodies to react with the immobilized antigen (Sample incubation). After washing to remove any unbound serum proteins, horseradish peroxidase conjugated anti-human IgG antibody 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 assay can be semi-quantified by measuring the reaction photometrically.
PRINCIPLE OF THE PROCEDURE (continued)

**Brief Assay Procedure**

- **<Sample incubation>** (20-30°C) 60 min.
  - Add 100 µL diluted sample (1:101) to each well of microwell plate.
  - Wash 4 times
- **<Conjugate incubation>** (20-30°C) 60 min.
  - Add 100 µL of Conjugate reagent to each well.
  - Wash 4 times
- **<Substrate incubation>** (20-30°C) 30 min.
  - Add 100 µL of Substrate to each well.
  - Add 100 µL of Stop solution to each well.
  - Read absorbance within 20 minutes.
  - Interpretation of result

**REAGENTS**

**BP230 MICROWELL STRIPS**
48 wells Microwell strips (6 x 8 well strips) coated with recombinant purified BP230-N and BP230-C antigen. The breakaway strips packed in a strip holder and sealed in a foil envelope with desiccant. Stable at 2-8°C until labeled expiration date.

**CALIBRATOR 1 (0 U/mL)**
One vial containing 1.5 mL of assay diluent including 0.09% sodium azide. Stable at 2-8°C until labeled expiration date.

**CALIBRATOR 2 (100 U/mL)**
One vial containing 1.5 mL of anti-BP230 positive antibody with assay diluent including 0.09% sodium azide. Stable at 2-8°C until labeled expiration date.

**CONJUGATE REAGENT**
One vial containing 8 mL of horseradish peroxidase conjugated goat anti-human IgG antibody. Stable at 2-8°C until labeled expiration date.

**ASSAY DILUENT**
One vial containing 50 mL of PBS, Tween 20 and 0.09% sodium azide. Stable at 2-8°C until labeled expiration date.

**WASH CONCENTRATE (10X)**
One vial containing 100 mL of PBS and Tween 20 as a 10X concentrate. Stable at 2-8°C until labeled expiration date.

**SUBSTRATE**
One vial containing 20 mL of 3,3',5,5'-tetramethylbenzidine dihydrochloride/hydrogen peroxide (TMB/H₂O₂). Stable at 2-8°C until labeled expiration date.

**STOP SOLUTION**
One vial containing 20 mL of 1.0N sulfuric acid. Stable at 2-8°C until labeled expiration date.

Note: The clinical laboratory should run 2 positive controls and 1 negative control with this test. These should include at least two different positive controls close to the cut-off value of the device. Since the controls are not included in the test kit, they could be from known positive and negative BP patients.

**WARNINGS AND PRECAUTIONS**

1. This product is for in vitro diagnostic use only.
2. Do not use kit components beyond the stated expiration dates.
3. 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 1.0N sulfuric acid, which is a poison and corrosive.

4. Calibrator 1, Calibrator 2 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 with plenty of water when disposing materials containing sodium azide into a drain.
WARNINGS AND PRECAUTIONS (continued)

5. The Conjugate reagent contains goat anti-human IgG antibody, which is from non-infectious animals. These components, however, should be treated as potential biohazards in use and for disposal.

6. Matching lot numbers of Microwell strips, Conjugate reagent, and Calibrator 2 must be used together in the assay. Do not substitute reagents from other kits.

7. All reagents must be brought to room temperature (20-30°C) before starting the assay.

8. Do not expose the kit to direct sun during storage or assay procedure.

9. Incubation temperatures above or below normal room temperature (20-30°C), shorter or longer time periods of incubation and inaccurate dilution may give erroneous results.

10. Avoid microbial and cross contamination of reagents or samples.

11. The wells must be rinsed with Wash solution properly to avoid a false positive result.

12. Carefully pipette each sample and reagent to avoid foaming and cross contamination between microwells.

13. 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.

14. Wash concentrate may become turbid at 2-8°C, which does not cause inconsistent results.

15. Instruments used for the test should be properly disposed or treated using the following method: Soak in 2% final conc. glutaraldehyde solution for more than 1 hour or soak in 0.5% Sodium hypochlorite solution (available chlorine: approx. 5,000ppm) for more than 1 hour or autoclave at 121°C for more than 20 minutes.

16. The BP230 antibodies value obtained from this assay are an aid to diagnosis only; each physician must interpret these results in light of the patient’s history, physical findings, and other diagnostic procedure.

LIMITATIONS

1. As with other diagnostic test procedures, the results obtained with the MESACUP BP230 ELISA Kit serve only as an aid to diagnosis and should not be interpreted as diagnostics in themselves.

2. A positive result indicates the presence of antibodies to recombinant BP230 which identify bullous pemphigoid and not other types of pemphigoid disease.

3. A negative result does not rule out pemphigoid disease: It is recommended that each patient be tested for BP180 antibodies.

PROCEDURE

MATERIALS REQUIRED BUT NOT PROVIDED

1. Microplate reader (wavelength: 450nm, 620 nm/reference)
2. Multichannel micropipette (e.g. 100 µL – 300 µL)
3. Single channel pipette (10 µL & 100 µL)
4. Autowasher or wash bottle
5. Deionized or distilled water
6. One liter graduated cylinder for preparation of Wash solution
7. Test tubes for patient sample dilutions (e.g.1,000 µL)
8. Disposable pipette tips
9. Paper towels
10. Microplate cover
11. Controls: positive and negative

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: The Wash concentrate must be diluted prior to use. Dilute the Wash concentrate 1:10 by adding 100 mL of the concentrate to 900 mL of distilled water. The diluted Wash solution is stable for 2 weeks at 2-8°C.
4. Do not dilute the other kit components which are ready-to-use.
PROCEDURE (continued)

PREPARATION OF SAMPLES

1. Use fresh patient sera. If storage is needed, they should be aliquoted and frozen below -20°C for up to one month, below -70°C for any longer storage. Do not repeat freezing and thawing.
   * In case of being stored below -20°C for more than 6 months or freezing and thawing repeatedly, nonspecific results are obtained because of IgG denaturation.

2. Dilute each patient serum 1:101 by adding 10 µL of serum to 1 mL of Assay diluent.
   * Diluted samples must be used within a day.

ASSAY PROCEDURE

1. SAMPLE INCUBATION
   Using the multi-channel pipetor, transfer 100 µL of Calibrator 1, Calibrator 2, positive controls, negative controls, and each diluted sample into the appropriate microwells of the antigen test plate. (Do not dilute Calibrators.)
   * Incubation starts on pipetting to the antigen-coated microwells. Pipetting should be completed as quickly as possible.
   Cover wells with a microplate cover and incubate for 60 minutes at room temperature (20-30°C).

2. WASHING
   Aspirate or discard the well contents. Fill the well with 300 µL of Wash solution and then completely aspirate or discard the contents. Wash 4 times.
   Tap the plate on a paper towel to remove any remaining Wash solution. When autowasher is used, wash 4 times.
   * Wash solution should be used at 20-30 °C.

3. CONJUGATE INCUBATION
   Add 100 µL of Conjugate reagent to each well with multichannel pipette.
   * Do not return the Conjugate reagent once taken out of vial.
   Cover wells with a microplate cover and incubate for 60 minutes at room temperature (20-30°C).

4. WASHING
   Wash the microplate following step 2

5. SUBSTRATE INCUBATION
   Add 100 µL of Substrate to each well with multichannel pipette.
   * Do not return the Substrate once taken out of vial.
   Cover wells with a microplate cover and incubate for 30 minutes at room temperature (20-30°C).

6. STOP REACTION
   Add 100 µL of the solution to each well with multichannel pipette.

READING

Read the absorbance of each well at 450 nm within 20 minutes. 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 within 20 minutes 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.

CALCULATION OF RESULT

Unit value (U/mL) = (A_{450}<Sample> - A_{450}<Calibrator 1>) / (A_{450}<Calibrator 2> - A_{450}<Calibrator 1>) x 100

*A_{450} is an abbreviation of absorbance value at 450 nm.
*An international reference material for anti-BP230 antibodies is not available; the assay is calibrated in relative arbitrary units.

QUALITY CONTROL

The positive control should be positive and the negative control should be negative. Each assay result should meet the following criteria.

\[
\begin{align*}
A_{450} \text{ of Calibrator 1:} & \leq 0.100 \\
A_{450} \text{ of Calibrator 2:} & \geq 0.500
\end{align*}
\]

If any of these are not met, the results are invalid and the test should be repeated. Before repeating assay, check the following procedures:

- Incubation temperature
- Incubation period of time
- Washing
- Dilutions

The user should run 2 positive controls and 1 negative control with each test. These should include at least two different positive controls close to the cut-off value of the device. These controls can be in-house controls prepared with established, reproducible values.
TEST INTERPRETATION AND EXPECTED VALUE

The following value was determined by ROC analysis with 72 BP samples and 109 normal samples.

The following is intended only as a guide for interpretation. Each laboratory is recommended to establish its own criteria for test interpretation based on sample populations typically encountered.

<table>
<thead>
<tr>
<th>Anti-BP230 value (U/mL)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 9</td>
<td>Negative for anti-BP230 Ab</td>
</tr>
<tr>
<td>≥ 9</td>
<td>Positive for anti-BP230 Ab</td>
</tr>
</tbody>
</table>

MESACUP BP230 ELISA Kit: Incidence of BP230 in Various Populations

<table>
<thead>
<tr>
<th>Population</th>
<th>No. Tested</th>
<th>No. Positive</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy blood donors (US)</td>
<td>82</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>Healthy blood donors (Japan)</td>
<td>109</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>Bullous pemphigoid (US)</td>
<td>69</td>
<td>48</td>
<td>69.6%</td>
</tr>
<tr>
<td>Bullous pemphigoid (Japan)</td>
<td>239</td>
<td>174</td>
<td>72.8%</td>
</tr>
<tr>
<td>Other autoimmune skin diseases (US) (1)</td>
<td>88</td>
<td>6</td>
<td>6.8%</td>
</tr>
<tr>
<td>Other autoimmune skin diseases (Japan) (2)</td>
<td>62</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>Infectious diseases (Japan) (3)</td>
<td>16</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>Other autoimmune diseases (Japan) (4)</td>
<td>28</td>
<td>0</td>
<td>0.0%</td>
</tr>
</tbody>
</table>

(1): pemphigus, linear IgA bullous dermatosis, epidermolysis bullous acquisita, bullous SLE
(2): pemphigus
(3): epstein barr virus, treponema pallidum
(4): SLE, SJS, RA, MCTD

PERFORMANCE CHARACTERISTICS

SPECIFICITY AND SENSITIVITY

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>No. Positive</th>
<th>Positive Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bullous pemphigoid (BP)</td>
<td>308</td>
<td>221</td>
<td>71.8%</td>
</tr>
<tr>
<td>Healthy Blood Donors</td>
<td>191</td>
<td>1</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

Sensitivity: 71.8% Specificity: 99.5%

COMPARATIVE STUDY

MESACUP BP230 ELISA

The following results were obtained using 68 healthy blood donors and 68 BP patients both tested using the IIF method and the MESACUP BP230 ELISA KITS.

<table>
<thead>
<tr>
<th>Predicate device (IIF)</th>
<th>Pos</th>
<th>Neg</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MESACUP BP230</td>
<td>46</td>
<td>1</td>
<td>47</td>
</tr>
<tr>
<td>ELISA KIT</td>
<td>17</td>
<td>72</td>
<td>89</td>
</tr>
</tbody>
</table>

Total: 63  73 136

95%CI

| Positive percent agreement | 73.4% | 0.60349 0.83429 |
| Negative percent agreement | 98.6% | 0.92602 0.99965 |
PERFORMANCE CHARACTERISTICS (continued)

COMPARATIVE STUDY (continued)

Overall percent agreement 86.8%  
Lower 0.79891  
Upper 0.91963

The MESACUP BP230 ELISA Kit has a 73.4% positive agreement rate when compared to the standard indirect immunofluorescence method. This is due to the fact that the IF method detects both BP180 and BP230 antibodies at the same time. When both MESACUP BP180 and BP230 ELISA Kit are performed on the same sample, the positive agreement with the IF method increases to 90%. These findings correlate with the results from Yoshida et. al. It is recommended that each patient is tested for both BP180 and BP230 antibodies for the most accurate diagnosis.

CROSS REACTIVITY

A cross reactivity study was performed using 150 samples of autoimmune skin diseases except BP, 16 infectious diseases samples, and 28 systemic autoimmune diseases samples.

Six (6) out of 150 autoimmune skin disease samples were positive, but there were no positive results for infectious and systemic autoimmune samples. Overall cross-reactivity of MESACUP BP230 ELISA Kit was 3.1%.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>No. Positive</th>
<th>Positive Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin autoimmune diseases</td>
<td>150</td>
<td>6</td>
<td>4.0%</td>
</tr>
<tr>
<td>Infectious and autoimmune</td>
<td>44</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>Total:</td>
<td>194</td>
<td>6</td>
<td>3.1%</td>
</tr>
</tbody>
</table>

PRECISION

Repeatability was demonstrated by testing 3 samples in 8 replicates. Reproducibility was determined by testing 3 samples on 5 different days (day-to-day) and by testing 3 samples in 3 lots (lot-to-lot). %CV values for reproducibility and repeatability were below 15% for each sample.

ASSAY RANGE

The assay range of this kit is from 5 U/mL to 150 U/mL. When the assay result exceeds 150 U/mL should report “over 150 U/mL”.

INTERFERING SUBSTANCES

Hemoglobin (up to 490 mg/dL), Bilirubin C (up to 19.7 mg/dL), chyle (up to 1,470 unit as Formazine) and/or Rheumatoid factor (up to 500 IU/mL) are not effective on the assay result, but avoid using highly hemolysed samples or highly lipemic samples.

BIBLIOGRAPHY