HEp-2 Immunoperoxidase Kit

For In-Vitro Research Use

Product Code: FK001.1P

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1 INTENDED USE

This indirect immunoperoxidase kit, with human epitheloma type 2 (HEp-2) cells as a substrate, is intended for use in the screening and titration of circulating antinuclear antibodies in human serum as an aid in the diagnosis and treatment of systemic lupus erythematosus (SLE).

2 SUMMARY AND EXPLANATION

Antinuclear antibody (ANA) is a term which describes a variety of autoantibodies against constituents of cell nuclei including DNA, RNA and various nuclear proteins (Ref 1). These ANA’s are found with cell nuclei including DNA, RNA and various nuclear proteins (Ref 1). These ANA’s are found with cell nuclei including DNA, RNA and various nuclear proteins (Ref 1). These ANA’s are found with cell nuclei including DNA, RNA and various nuclear proteins (Ref 1). These ANA’s are found with cell nuclei including DNA, RNA and various nuclear proteins (Ref 1). These ANA’s are found with cell nuclei including DNA, RNA and various nuclear proteins (Ref 1).

Frozen sections of rat liver were historically the most popular substrate for demonstration of ANA’s but these have now been replaced by various types of cell lines. HEp-2 cells (Ref 3) are an epithelial cell line derived from a human carcinoma of the larynx. HEp-2 cells are characterized by extremely large nuclei compared to other cell lines. When used in ANA screening the advantages of HEp-2 cells are highly visible to the naked eye. HEp-2 cells are larger than normal cells and enable easier visualisation of cell morphology with a consequent increase in assay sensitivity over rat liver sections and finally, many HEp-2 cells are actively dividing, exposing antigens not normally expressed in the resting cells of rat liver sections (Ref 3).

3 PRINCIPLE

An indirect immunoperoxidase technique is utilised where patient samples and appropriate controls are incubated with the HEp-2 substrate. The unreacted antibodies are washed off and then an appropriate peroxidase labelled conjugate is applied. Unbound conjugate is washed off before. Peroxidase enzyme is demonstrated using 3- amino-9-ethylcarbazole (AEC) as a substrate. Slides are viewed with a bright field microscope and positive samples are revealed by red staining which corresponds to areas of the HEp-2 cell where autoantibody has bound.

4 REAGENTS

1. HEp-2 cells on 5-well slides, individually wrapped in foil bags contains a dessicant. The specially treated slides ensure sample containment within the test area.
2. Positive control serum giving an homogeneous pattern on HEp-2 slides. Provided prediluted and ready to use in a dropper bottle.
3. Negative control serum, universally negative for all autoantibodies. Provided prediluted and ready to use in a dropper bottle.
4. Affinity purified sheep anti-human IgG (H&L), optimally labelled with peroxidase enzyme.
5. AEC / DMP.
6. Acetate buffer pH5.0.
7. Hydrogen peroxide (30%).
8. Phosphate buffered saline (PBS), provided as a 20-fold concentrate in liquid form.
9. Blotters, designed to fit around all wells and are used to blot the slides after washing.
10. Mounting medium, glycerol jelly.
11. Coverslips (22 x 70mm), provided to mount slides prior to microscopic examination.

5 CAUTION

All donors of human serum supplied have been tested and found to be negative for Hepatitis B surface antigen and antibodies to Hepatitis C virus and Human Immunodeficiency Virus (HIV 1 & 2). However, these tests cannot guarantee the absence of infective agents. Proper handling and disposal methods should be established as for all potentially infective material and only personnel adequately trained in such methods should be permitted to perform the procedures.

The kit controls contain 0.1% sodium azide as a preservative and must be handled with caution – do not ingest or allow contact with the skin or mucous membranes. If contact does occur wash with a large volume of water and seek medical advice. Explosive metal azides may be formed with the lead and copper plumbing; on disposal of reaction, flush with a large volume of water to prevent azide build up.

This product should only be used by suitably trained persons for the purposes stated. Adherence to the given procedure is recommended. Reagents from different batch numbers of kits are not interchangeable. If large numbers of tests are performed care should be taken to ensure that all reagents are from the same batch.

AEC/DMP is both harmful, toxic and a suspected carcinogen and must be handled with extreme care (using appropriate protective clothing eg. Gloves, mask etc). Safety data sheets are available on request.

6 STORAGE AND STABILITY

Unopened kits or slides should be stored at 2-8°C and can be used until the given expiry date. DO NOT FREEZE. Once slides are removed from a foil bag, they should be used immediately. Diluted PBS buffer can be stored for up to one month at 2-8°C. All reagents should be stored at 2-8°C.

7 SPECIMEN COLLECTION

Blood samples should be collected by venepuncture, allowed to clot naturally and the serum separated as soon as possible to prevent haemolysis. The serum may be stored at 2-8°C for up to 7 days prior to analysis (Ref 4), or for prolonged storage, adjusted and stored at -20°C or below. DO NOT freeze and thaw sera more than once. Avoid using isopaenic, haemocytoblastic or microbially contaminated sera as decreased titres or unclear staining patterns may occur.

8 PROCEDURE

8.1 Materials Provided

<table>
<thead>
<tr>
<th>Product Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>S07 kit FK001.1P</td>
<td>1. 10 x 5-well HEp-2 substrate slides</td>
</tr>
<tr>
<td>1. 1 x 1mL prediluted positive control</td>
<td></td>
</tr>
<tr>
<td>1. 1 x 1mL prediluted negative control</td>
<td></td>
</tr>
<tr>
<td>1. 1 x 200mL anti-human IgG (H&amp;L) peroxidase conjugate</td>
<td></td>
</tr>
<tr>
<td>5. 2 x 60mL PBS concentrate (x20)</td>
<td></td>
</tr>
<tr>
<td>6. 1 x 2mL AEC/DMP</td>
<td></td>
</tr>
<tr>
<td>7. 1 x 3mL Hydrogen peroxide</td>
<td></td>
</tr>
<tr>
<td>8. 2 x 10mL 50mM Acetyl buffer pH5.0</td>
<td></td>
</tr>
<tr>
<td>10. 30 x blotters</td>
<td></td>
</tr>
<tr>
<td>11. 10 x coverslips (22 x 70mm)</td>
<td></td>
</tr>
<tr>
<td>12. 1 x instruction leaflet</td>
<td></td>
</tr>
</tbody>
</table>

8.2 Additional Materials Required

1. Distilled water to dilute the PBS concentrate
2. Container for PBS buffer
3. Micropipettes and disposable tips to apply patient samples
4. Humid chamber for incubation steps
5. Bright field microscope
6. Plastic squeeze bottle for initial wash in PBS

8.3 Test Procedure

1. Dilute PBS concentrate

Dilute PBS concentrate with distilled water and mix. The PBS is used for diluting patient samples and as a wash buffer. Cover and store for up to one month 2-8°C. The PBS is used for diluting patient samples and as a wash buffer.

2. Dilute patient samples

A. Screening: Dilute patient samples 1/40 by adding 25µL of serum to 975µL of PBS buffer.

B. Titration: Make serial dilutions of positive screened samples with PBS buffer (eg. 1/40, 1/80, 1/160, 1/320 and 1/640 etc.)

3. Substrate Slides

Allow substrate slides to reach room temperature (18°C-28°C) for approximately 30 minutes prior to removal from pouches. Label slides appropriately, place in the humid chamber and add one drop of positive and negative control to wells 1 and 2 respectively. Add 25µL of diluted patient samples to the remaining wells.

4. Slides Incubation

Incubate slides for 30 minutes in a humid chamber at room temperature (18°C - 28°C). (Mistened paper towels at the bottom of the chamber will maintain humidity).

5. PBS Wash

Remove slides from humid chamber and rinse briefly with PBS in a squeeze bottle. Do not agitate directly on the slides. Place slides in a rack and rinse pre and agitation or stir for 10 minutes. Dilute peroxidase conjugate 1/400 in PBS as required. Store unused conjugate undiluted.

6. Addition of Peroxidase conjugate

Shake off excess PBS and blot around wells using blotters provided or filter paper/tissues etc. Return slides to humidity chamber and incubate each well with 50µL of conjugate. DO NOT LEAVE WELLS UNCOVERED FOR LONGER THAN 15 SECONDS. DRYING OUT OF THE SUBSTRATE SERIOUSLY AFFECTS THE RESULTS.

7. Slide Incubation

Incubate slides for 30 minutes in humid chamber at room temperature (18°C - 28°C) in the dark.

8. PBS Wash

Wash again as described in step 5.

9. Substrate preparation:Incubation

WEAR GLOVES WHEN PREPARING THIS SUBSTRATE AND MAKE ALL DILUTIONS IN GLASS VESSELS.

Add 100µL of AEC/DMP to 900µL of Acetyl buffer pH5.0, followed by 1%L of 3% hydrogen peroxide immediately before use. Mix well. Add 100µL of substrate to each well and incubate for 30 minutes (sufficient substrate components are provided for the development of all slides in the kit).

10. PBS Wash

Wash in PBS, as in step 5. Melt glycerine jelly mountant by placing dropper bottle in water bath at 50-60°C.

11. Mounting with coverslip

Remove one slide at a time from PBS wash. Quickly dry around the wells and add a drop of melted mounting medium to each well. Carefully lower the slide onto the coverslip, avoiding air bubbles, but if present do not attempt to remove. Wipe excess medium from around edge of coverslip.

12. View slides under a microscope

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## 9 RESULTS

### Quality Control

Positive and negative controls should be used every time a batch of samples are tested. The positive control should give a red homogenous staining pattern in cell nuclei. The negative control should show minimal staining in all HEp-2 cells, with no discernible red colouration. If the controls do not appear as described, the test is invalid and should be repeated.

### Interpretation of Results

#### Negative

A sample is considered negative if specific nuclear staining is equivalent to or less than the negative control well. Samples may exhibit background staining but should be reported as negative (Ref 4).

#### Positive

A sample is considered positive if nuclear staining is observed to be greater than the negative control well, and a clearly discernible pattern can be seen in most of the HEp-2 cells.

### PATTERN APPEARANCE ANTIGENS INVOLVED MAIN DISEASE ASSOCIATIONS

<table>
<thead>
<tr>
<th>Homogeneous</th>
<th>Solid nucleus staining</th>
<th>ds DNA, rDNA, Histones</th>
<th>High titres: SLE Low titres: SLE or other connective tissue disorders (Ref 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speckled</td>
<td>Fine or granular (coarse) speckles, usually without staining of the nucleus</td>
<td>SS-A, SS-B, RNP, Sm, Scl-70 + others</td>
<td>High titres: Sjogrens-sicca complex syndrome SLE Mixed connective tissue disease Scleroderma Low titres: other connective tissue diseases</td>
</tr>
<tr>
<td>Nucleolar</td>
<td>Large coarse speckles (usually less than 6 speckles per nucleus, with or without fine speckles</td>
<td>RNP, Sm, Scl-70 + others</td>
<td>High titres: Scleroderma &amp; Sjogren’s syndrome (Ref 6)</td>
</tr>
<tr>
<td>Centromere</td>
<td>Discrete speckles (usually a multiple of 46)</td>
<td>Chromosomal Centromere</td>
<td>CREST Syndrome (Ref 7)</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>Coarse granular filamentous cytoplasmic speckled pattern extending around the nucleus and throughout the cytoplasm</td>
<td>M2</td>
<td>Primary biliary cirrhosis (common) Scleroderma (40%) and occasionally in overlap syndromes</td>
</tr>
</tbody>
</table>

NB: Each laboratory should establish at which point a positive result is considered clinically significant.

## 10 LIMITATIONS OF PROCEDURE

1. This test alone should not be considered diagnostic. All other factors including the clinical history of the patients and other serological or biopsy results must also be taken into account.

2. Suitability for use with other manufacturers’ IFA reagents has not been assessed but use with such reagents should not necessarily be excluded.

3. Drug induced SLE patients may have positive homogenous or peripheral ANA’s.

4. A small percentage of SLE patients may be ANA negative by indirect immunofluorescence/immunoperoxidase but may have ANA’s by other methods (Ref 7).

5. High-titred ANA is highly suggestive of connective tissue disease but should be considered with all other serological results and the patient’s clinical history, before being considered diagnostically significant.

6. Staining patterns often change as the sample is titred out to end point. This is usually due to the presence of more that one anti-nuclear antibody.

7. Positive ANA’s are also seen in a small proportion of patients with infectious and/or neoplastic diseases.

8. Pattern recognition of autoantibody specificity (except for nuclear and centromere patterns) should be carefully considered. Combinations of autoantibodies can induce homogenous or speckled patterns, and it is recommended that specific testing for dsDNA and ENAs be performed on all speckled or homogenous samples.

## 11 REFERENCES


