QUANTA Lite™ M2 EP (MIT3) ELISA Bulk Pack 704542

For In Vitro Diagnostic Use
CLIA Complexity: High

Principles of the Procedure
Affinity-purified recombinant antigen (MIT3) containing immunodominant portions of PDC-E2, BCOADC-E2, and OGDC-E2 is bound to the wells of a polystyrene microwell plate under conditions that will preserve the antigen in its native state. Pre-diluted controls and diluted patient sera are added to separate wells, allowing any mitochondrial antibodies present to bind to the immobilized antigen. Unbound sample is washed away and an enzyme labeled anti-human IgG conjugate is added to each well. A second incubation allows the enzyme labeled anti-human IgG to bind to any patient antibodies, which have become attached to the microwells. After washing away any unbound enzyme labeled anti-human IgG, the remaining enzyme activity is measured by adding a chromogenic substrate and measuring the intensity of the color that develops. The assay can be evaluated spectrophotometrically by measuring and comparing the color intensity that develops in the patient wells with the color in the control wells.

QUANTA Lite™ M2 EP (MIT3) ELISA is an enzyme-linked immunosorbent assay (ELISA) for the semi-quantitative detection of mitochondria antibodies in human serum. The presence of mitochondria antibodies can be used in conjunction with clinical findings and other laboratory tests to aid in the diagnosis of primary biliary cirrhosis.

Primary biliary cirrhosis (PBC) is a chronic liver disease characterized by the destruction of the small intrahepatic bile ducts. Progressive duct destruction leads to increasing functional impairment of the liver and, over time, can lead to liver failure and the necessity of liver transplantation. 1,2 The etiology of PBC is unknown, although a genetic component as well as other factors may be important for the development of the disease. 1,3,4

PBC typically occurs between the ages of 30 and 65 and affects women more frequently than men (estimated female: male ratio of 9:1). 3,4 The prevalence of PBC in first-degree relatives of PBC patients ranges from 1.3 to 6.4%. 4,5 PBC is found in all races and has a worldwide distribution. Wide variations in geographic prevalence of PBC have been reported, from estimates of 2 per 100,000 in Japan and Australia to 40 per 100,000 in the United States. 3,6

Serological assays are important aids to the recognition and diagnosis of PBC since many antibodies associated with PBC are present before symptoms become evident. 7,8 Anti-mitochondrial antibodies (AMA), detected by indirect immunofluorescence assay (IFA), are the classic serological marker of PBC. 10 Although AMA have been reported in up to 90-95% of PBC patients, the frequency of detection can be significantly lower. 11 Detection of AMA by IFA is highly dependent on observer skill, technical components of the assay, and the presence of other antibodies which can obscure or confuse the interpretation of the IFA patterns.

Early studies described 9 subtypes of mitochondrial antigens, termed M1-M9.12 The major autoantigens targeted by PBC patient sera recognize the M2 antigen fraction. The primary components of the M2 antigen were found to be members of the 1-oxo-acid dehydrogenase complex. The specific antigens were identified as the E2 subunits of the pyruvate dehydrogenase complex (PDC-E2), the branched-chain 2-oxo-acid dehydrogenase complex (BCOADC-E2), and the 2-oxo glutarate dehydrogenase complex (OGDC-E2).13 Identification of these antigens permitted the development of ELISA assays. ELISA tests have been shown to be more sensitive than IFA. 14,15 First generation anti-M2 ELISA tests utilized PDC-E2 as the primary substrate to detect PBC-specific antibodies. While 80-90% of histologically proven PBC patients have anti-PDC-E2 antibodies, about 10% of PBC patients only react to BCOADC-E2 and/or OGDC-E2. 16,17 Gershwin and Leung developed and patented a triple expression hybrid clone (“MIT3”) which expresses the immunodominant epitopes of PDC-E2, BCOADC-E2, and OGDC-E2. 15,16,19 The MIT3-based ELISA was shown to have enhanced performance over IFA or conventional PDC-E2- based ELISA tests and detected AMA in over two-thirds of the sera from “AMA-negative” (by IFA) PBC patients. 15,16 Since the presence of AMA can precede the development of symptomatic disease, the ability to more accurately identify the presence of markers for PBC can contribute to earlier diagnosis, treatment and may slow the progression of the disease. 7,8
Reagents
1. Seven Polystyrene microwell ELISA plates coated with purified recombinant MIT3 antigen, (12-1 x 8 wells), with holder in foil package containing desiccants
2. ELISA Negative Control, 7 vials of buffer containing preservative and human serum with no human antibodies to Mitochondria M2, prediluted, 1.2mL
3. M2 EP (MIT3) ELISA Low Positive, 7 vials of buffer containing preservative and human serum antibodies to Mitochondria M2, prediluted, 1.2mL
4. M2 EP (MIT3) ELISA High Positive, 7 vials of buffer containing preservative and human serum antibodies to Mitochondria M2, prediluted, 1.2mL
5. HRP Sample Diluent, 7 vials – colored pink containing Tris-buffered saline, Tween 20, protein stabilizers and preservative, 50mL
6. HRP Wash Concentrate, 7 vials of 40x concentrate - colored red containing Tris-buffered saline and Tween 20, 25mL. Refer to the Methods Section for dilution instructions.
7. HRP IgG Conjugate, (goat), anti-human IgG, 7 vials – colored blue containing buffer, protein stabilizers and preservative, 10mL
8. TMB Chromogen, 7 vials containing stabilizers, 10mL
9. HRP Stop Solution, 0.344M Sulfuric Acid, 7 vials – colorless, 10mL

Materials provided
7 M2 EP (MIT3) ELISA microwell plates (12-1 x 8 wells), with holder
7 1.2mL prediluted ELISA Negative Control
7 1.2mL prediluted M2 EP (MIT3) ELISA Low Positive
7 1.2mL prediluted M2 EP (MIT3) ELISA High Positive
7 50mL HRP Sample Diluent
7 25mL HRP Wash Concentrate, 40x concentrate
7 10mL HRP IgG Conjugate, (goat), anti-human IgG
7 10mL TMB Chromogen
7 10mL HRP Stop Solution, 0.344M Sulfuric Acid

Additional Materials Required But Not Provided
Micropipets to deliver 5, 100, 200-300 and 500µL
Disposable micropipet tips
Test tubes for patient sample dilutions, 4mL volume
Distilled or deionized water
1L container for diluted HRP Wash Concentrate
Microwell plate reader capable of measuring OD at 450nm (and 620nm for dual wavelength readings)

Specimen
This procedure should be performed with a serum specimen. Addition of azide or other preservatives to the test samples may adversely affect the results. Microbially contaminated, heat-treated, or specimens containing visible particulate should not be used. Grossly hemolyzed, icteric or lipemic serum or specimens should be avoided.
Following collection, the serum should be separated from the clot. NCCLS Document H18-A3 recommends the following storage conditions for samples: 1) Store samples at room temperature no longer than 8 hours. 2) If the assay will not be completed within 8 hours, refrigerate the sample at 2-8°C. 3) If the assay will not be completed within 48 hrs, or for shipment of the sample, freeze at -20°C or lower. Frozen specimens must be mixed well after thawing and prior to testing.

Special Safety Precautions/Storage Conditions
1. Store all the kit reagents at 2-8°C. Do not freeze. Reagents are stable until the expiration date when stored and handled as directed.
2. Unused antigen coated microwell strips should be resealed securely in the foil pouch containing desiccants and stored at 2-8°C.
3. Diluted wash buffer is stable for 1 week at 2-8°C.

Procedural Notes:
Warnings
1. WARNING: This product contains a chemical (0.02% chloramphenicol) in the sample diluent, controls, and conjugate known to the State of California to cause cancer.
2. All human source material used in the preparation of controls for this product has been tested and found negative for antibody to HIV, HbsAg, and HCV by FDA cleared methods. No test method however can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, the M2 EP (MIT3) ELISA Low Positive, M2 EP (MIT3) ELISA High Positive and ELISA Negative Control should be handled in the same manner as potentially infectious material.20
3. Sodium Azide is used as a preservative. Sodium Azide is a poison and may be toxic if ingested or absorbed through the skin or eyes. Sodium azide may react with lead or copper plumbing to form potentially explosive metal azides. Flush sinks, if used for reagent disposal, with large volumes of water to prevent azide build-up.

4. The HRP conjugate contains a dilute poisonous/corrosive chemical, which may be toxic if ingested in large amounts. To prevent possible chemical burns, avoid contact with skin and eyes.

5. TMB Chromogen contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.

6. The HRP Stop Solution consists of a dilute sulfuric acid solution. Avoid exposure to bases, metals, or other compounds, which may react with acids. Sulfuric acid is a poison and corrosive, which may be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.

7. Use appropriate personal protective equipment while working with the reagents provided.

8. Spilled reagents should be cleaned up immediately. Observe all federal, state and local environmental regulations when disposing of wastes.

**Precautions**

1. This product is for In Vitro Diagnostic Use.
2. Substitution of components other than those provided in this system may lead to inconsistent results.
3. Incomplete or inefficient washing and insufficient liquid removal from the ELISA well strips will cause poor precision and/or high background.
4. Adaptation of this assay for use with automated sample processors and other liquid handling devices, in whole or in part, may yield differences in test results from those obtained using the manual procedure. It is the responsibility of each laboratory to validate that their automated procedure yields test results within acceptable limits.
5. A variety of factors influence the assay performance. These include the starting temperature of the reagents, the ambient temperature, the accuracy and reproducibility of the pipetting technique, the thoroughness of washing and liquid removal from the wells of the ELISA strips, the photometer used to measure the results, and the length of the incubation times during the assay. Careful attention to consistency is required to obtain accurate and reproducible results.
6. Strict adherence to the protocol is recommended.
7. Incomplete resealing of the zip-lock pouch containing microwell strips and desiccants will result in antigen degradation and poor precision.
8. Unacceptably low absorbencies may be observed following two or more uses from a single bottle of HRP conjugate over a period of time. It is important to follow all recommended HRP conjugate handling procedures to prevent this occurrence.
9. Chemical contamination of the HRP conjugate can result from improper cleaning or rinsing of equipment or instruments. Residues from common laboratory chemicals such as formalin, bleach, ethanol or detergent will cause degradation of the HRP conjugate over time. Thoroughly rinse all equipment or instruments after the use of chemical cleaners/disinfectants.

**Quality Control**

1. The M2 EP (MIT3) ELISA Low Positive, the M2 EP (MIT3) ELISA High Positive and the ELISA Negative Control should be run with every batch of samples to ensure that all reagents and procedures perform properly.
2. Note that since the M2 EP (MIT3) ELISA Low Positive, the M2 EP (MIT3) ELISA High Positive and the ELISA Negative Control are prediluted, they do not control for procedural methods associated with dilution of specimens.
3. Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations. Additional suitable control sera may be prepared by aliquoting pooled human serum specimens and storing at $\leq -20^\circ$C.
4. In order for the test results to be considered valid, all of the criteria listed below must be met. If any of these are not met, the test should be considered invalid and the assay repeated.
   a. The absorbance of the prediluted M2 EP (MIT3) ELISA High Positive must be greater than the absorbance of the prediluted M2 EP (MIT3) ELISA Low Positive, which must be greater than the absorbance of the prediluted ELISA Negative Control.
   b. The prediluted M2 EP (MIT3) ELISA High Positive must have an absorbance greater than 1.0 while the prediluted ELISA Negative Control absorbance cannot be over 0.2.
   c. The M2 EP (MIT3) ELISA Low Positive absorbance must be more than twice the ELISA Negative Control or over 0.25.
   d. The ELISA Negative Control and M2 EP (MIT3) ELISA High Positive are intended to monitor for substantial reagent failure. The M2 EP (MIT3) ELISA High Positive will not ensure precision at the assay cutoff.
   e. The user should refer to NCCLS Document C24-A2 for additional guidance on appropriate QC practices.21
Procedure:

Method Before you start
1. Bring all reagents and samples to room temperature (20-26°C) and mix well.
2. Dilute the HRP Wash Concentrate 1:40 by adding the contents of the HRP Wash Concentrate bottle to 975mL of distilled or deionized water. If the entire plate will not be run within this period, a smaller quantity can be prepared by adding 2.0mL of the concentrate to 78mL of distilled or deionized water for every 16 wells that will be used. The diluted buffer is stable for 1 week at 2-8°C.
3. Prepare a 1:101 dilution of each patient sample by adding 5µL of sample to 500µL of HRP Sample Diluent. Diluted samples must be used within 8 hours of preparation. **DO NOT DILUTE** the M2 EP (MIT3) ELISA Low Positive, M2 EP (MIT3) ELISA High Positive and ELISA Negative Control.
4. Determination of the presence or absence of antibodies to M2 (MIT3) using arbitrary units requires two wells for each of the three controls and one or two wells for each patient sample. It is recommended that samples be run in duplicate.

Assay Procedure
1. **ALL REAGENTS MUST BE BROUGHT TO ROOM TEMPERATURE (20-26°C) PRIOR TO BEGINNING THE ASSAY.** Place the required number of microwells/strips in the holder. Immediately return unused strips to the pouch containing desiccants and seal securely to minimize exposure to water vapor.
2. Add 100µL of the prediluted M2 EP (MIT3) ELISA Low Positive, the M2 EP (MIT3) ELISA High Positive, the ELISA Negative Control and the diluted patient samples to the wells. Cover the wells and incubate for 30 minutes at room temperature on a level surface. The incubation time begins after the last sample addition.
3. Wash step: Thoroughly aspirate the contents of each well. Add 200-300µL of the diluted HRP Wash buffer to all wells then aspirate. Repeat this sequence twice more for a total of three washes. Invert the plate and tap it on absorbent material to remove any residual fluid after the last wash. It is important to completely empty each well after each washing step. Maintain the same sequence for the aspiration as was used for the sample addition.
4. Add 100µL of the HRP IgG Conjugate to each well. Conjugate should be removed from the bottles using standard aseptic conditions and good laboratory techniques. Remove only the amount of conjugate from the bottle necessary for the assay. **TO AVOID POTENTIAL MICROBIAL AND/OR CHEMICAL CONTAMINATION, NEVER RETURN UNUSED CONJUGATE TO THE BOTTLE.** Incubate the wells for 30 minutes as in step 2.
5. Wash step: Repeat step 3.
6. Add 100µL of TMB Chromogen to each well and incubate in the dark for 30 minutes at room temperature.
7. Add 100µL of HRP Stop Solution to each well. Maintain the same sequence and timing of HRP Stop Solution addition as was used for the TMB Chromogen. Gently tap the plate with a finger to thoroughly mix the wells.
8. Read the absorbance (OD) of each well at 450nm within one hour of stopping the reaction. If bichromatic measurements are desired, 620nm can be used as a reference wavelength.

Reporting Results:

Interpretation of Results
The ELISA assay is very sensitive to technique and is capable of detecting even small differences in patient populations. The values shown below are suggested values only. Each laboratory should establish its own normal range based upon its own techniques, controls, equipment and patient population according to their own established procedures. The sample can then be classified as negative, equivocal or positive according to the table below.

<table>
<thead>
<tr>
<th>Units</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>≤ 20</td>
</tr>
<tr>
<td>Equivocal</td>
<td>20.1-24.9</td>
</tr>
<tr>
<td>Positive</td>
<td>≥ 25</td>
</tr>
</tbody>
</table>

1. A positive result indicates the presence of Mitochondria antibodies and suggests the possibility of primary biliary cirrhosis.
2. A negative result indicates no Mitochondria antibody or levels below the negative cut off of the assay.
3. It is suggested that the results reported by the laboratory should include the statement: “The following results were obtained with the INOVA QUANTA Lite™ M2 EP (MIT3) ELISA. M2 EP (MIT3) values obtained with different manufacturers’ assay methods may not be used interchangeably. The magnitude of the reported IgG levels cannot be correlated to an endpoint titer.”

Calculations
The average OD for each set of duplicates is first determined. The reactivity for each sample can then be calculated by dividing the average OD of the sample by the average OD of the M2 EP (MIT3) ELISA Low Positive. The result is multiplied by the number of units assigned to the M2 EP (MIT3) ELISA Low Positive found on the label.

\[
\text{Sample Value} = \frac{\text{Sample OD}}{\text{M2 EP (MIT3) ELISA Low Positive OD}} \times \text{M2 EP (MIT3) ELISA Low Positive (units)}
\]
Reactivity is related to the quantity of antibody present in a non-linear fashion. While increases and decreases in patient antibody concentrations will be reflected in a corresponding rise or fall in reactivity, the change is not proportional (i.e. a doubling of the antibody concentration will not double the reactivity). If a more accurate quantitation of patient antibody is required, serial dilutions of the patient sample should be run and the last dilution to measure positive in the assay should be reported as the patient's antibody titer.

Expected Values & Specific Performance Characteristics

The prevalence of PBC ranges from estimates of 2 per 100,000 in Japan and Australia to 40 per 100,000 in the United States.\textsuperscript{3, 6} While anti-mitochondrial antibodies have been generally thought to be present in 90-95% of PBC patients, the frequency of detected antibodies depends on the cohort studied and the sensitivity may be as low as 72%.\textsuperscript{11} The specificity of AMA by IFA for PBC is in the range of 95-97%\textsuperscript{11}.

Normal Range

Anti-M2 (MIT3) antibodies in Asymptomatic Healthy Individuals

A panel of 520 asymptomatic, healthy individuals was tested for M2 EP(MIT3) antibodies with the QUANTA Lite\textsuperscript{TM} M2 EP (MIT3) ELISA. Age and sex data was available for 299 of the specimens. The ages ranged from 18-78 years old and included 150 male and 149 female individuals. The average value for this population was 7.6 units, the median value was 6.0 units. The specificity of the assay was 98.5% (512/520) for the normal subjects. Two reactive specimens showed clear AMA patterns by IFA examination.

Specific Performance Characteristics

The frequency of M2 (MIT3) antibodies detected with the QUANTA Lite\textsuperscript{TM} M2 EP (MIT3) ELISA on a total of 980 definite PBC (978) or PBC/Autoimmune Hepatitis (AIH) (2) specimens assembled from several clinical cohorts is presented in Table 1. Specimens with AIH, known AMA-negative PBC or patients with suspected, but unconfirmed disease were excluded. The overall sensitivity of the assay was 87.3% (856/980). The specificity of the assay was 98.7% (590/598). The positive predictive value was 99.1% and the negative predictive value was 82.6%. The mean and median values for all non-PBC or PBC/AIH were 7.5 and 5.9 units respectively.

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>n=</th>
<th>pos</th>
<th>equiv</th>
<th>neg</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBC (967) or PBC/AIH (13)</td>
<td>980</td>
<td>856</td>
<td>2</td>
<td>122</td>
</tr>
<tr>
<td>healthy controls (520); Primary Sclerosing Cholangitis-PSC (47), other disease controls (31)</td>
<td>598</td>
<td>8</td>
<td>4</td>
<td>586</td>
</tr>
</tbody>
</table>

Sensitivity: 87.3% (856/980); 95% Confidence Interval (CI): 85.1% to 89.4%
Specificity: 98.7% (590/598); 95% CI: 97.4% to 99.4%

Cross Reactivity

Sera from 155 patients with non-PBC liver disease (70 AIH, 46 PSC, 8 AIH/PSC) and autoimmune or infectious disease (3 LKM-1, 2 SLA, 3 GPA, 3 chromatin, 5 ASCA, 2 Sm, 2 RNP, 2 SS-A, 2 SS-B, 2 Scl-70, 2 J o-1, 1 SLE, 2 HCV, 2 suspected non-PBC liver disease) were tested with the QUANTA Lite\textsuperscript{TM} M2 EP (MIT3) ELISA to assess the assay’s specificity. None of the autoimmune or infectious disease sera were interpreted as positive by the QUANTA Lite\textsuperscript{TM} M2 EP(MIT3) ELISA. Five AIH specimens were positive, but these may have an undiagnosed or evolving AIH/PBC syndrome. All AIH or AIH/PSC specimens were excluded from the calculation of specificity.

Precision and Reproducibility

Intra-assay performance was assessed by running 6 specimens a total of 6 times each

| Table 2: Intra-assay Performance of QUANTA Lite\textsuperscript{TM} M2 EP(MIT3) ELISA |
|------------------|-----|-----|-----|-----|-----|-----|
|                  | A   | B   | C   | D   | E   | F   |
| Mean unit        | 123.8 | 7.3 | 55.4 | 11.8 | 168.2 | 8.1 |
| SD               | 3.46 | 0.95 | 3.14 | 0.96 | 2.55 | 0.78 |
| CV %             | 2.8  | 13.0 | 5.7  | 8.1  | 1.5  | 9.7 |

Inter-assay performance was assessed by testing in duplicate, 5 specimens, the kit high positive control (HPC) and negative control (NC) twice daily (once in the morning and once in afternoon) for 3 days.

| Table 3: Inter-assay Performance for QUANTA Lite\textsuperscript{TM} M2 EP (MIT3) ELISA |
|------------------|-----|-----|-----|-----|-----|-----|
|                  | HPC | NC  | A   | B   | C   | D   |
| Mean units       | 112.7 | 2.2 | 110.3 | 7.8 | 54.4 | 11.6 |
| SD               | 2.98 | 0.16 | 2.56 | 1.27 | 1.57 | 0.61 |
| CV %             | 2.6  | 7.4  | 2.3  | 16.2 | 2.9  | 5.3  |
|                  |     |     |     |     |     |     |

NCCLS 704542
Page 5 of 6
Relative Specificity/Sensitivity
Precision - N/A
Correlation – N/A

Limitations of the Procedure
1. The presence of immune complexes or other immunoglobulin aggregates in the patient sample may cause an increased level of non-specific binding and produce false positives in this assay.
2. Not all primary biliary cirrhosis patients are positive for mitochondrial antibodies.
3. Results of this assay should be used in conjunction with clinical findings and other serological tests.
4. The assay performance characteristics have not been established for matrices other than serum.

References