### 3.0 PRINCIPLE

A Sequential CLIA Method (TYPE 1):

The reagents required for the sequential CLIA assay include immobilized antigen that forms an antibody and enzyme-linked species-specific antibody. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenous biotinylated H. Pylori antigen.

Upon mixing biotinylated antigen and a serum containing the antibody, reaction results between the antigen and the antibody to form an immune complex. The interaction is illustrated by the following equation:

\[ h-Ab(X-H_Pylori) + BtnAg(H_Pylori) \rightarrow Ab(X-H_Pylori) - BtnAg(H_Pylori) \]

\[ ^{2}Ag(s) = Biotinylated \\text{(Antigen Constant)} \]
\[ h-Ab(X-H_Pylori) = Human \\text{Auto-Antibody (Variable Quantity)} \]
\[ Ab(X-H_Pylori) = Antibody (Variable Quantity) \]
\[ k_a = Rate \\text{Constant of Association} \]
\[ k_d = Rate \\text{Constant of Dissociation} \]

Similarly, the complex is deposed to the well through the high affinity reaction of streptavidin and biotinylated antigen. This interaction is illustrated by the following:

\[ h-Ab(X-H_Pylori) + BtnAg(H_Pylori) \rightarrow Ab(X-H_Pylori) - BtnAg(H_Pylori) \]

The anti-h-IgG, IgM or IgA enzyme conjugate that binds to the immune complex in a second incubation is separated from unreacted species by washing the microtiter plate. The activity in this fraction is directly proportional to the antibody concentration in the specimen. By utilizing several different serum references of known antibody activity, a reference curve can be generated from which the antibody activity of an unknown can be ascertained.

### 4.0 REAGENTS

#### Materials Provided:

- **A. Anti-H-Pylori Calibrators – 5ml/vial - Icos A-E**
  - Five (5) vials of calibrators for anti-H-Pylori at levels of 0(A), 10(B), 25(C), 50(D), and 100(E) U/ml of the IgG, IgM or IgA type. Store at 2-8°C. A preservative has been added. (Manufactured Reference Value: 10 U/ml)
- **B. H-Pylori Biotin Reagent – 10ml/vial - Icon V**
  - One (1) vial of biotinylated isolated H. Pylori IgG, IgM or IgA in a buffering matrix. A preservative has been added. Store at 2-8°C.
- **C. H-Pylori Antigen Tracer Reagent – 10ml/vial - Icon C**
  - One (1) vial of anti-human IgG, IgM or IgA-horseradish peroxidase (HRP) conjugate in a buffering matrix. A preservative has been added. Store at 2-8°C.
- **D. H-Pylori Reactives Wells A-C**
  - Nine (9) wells coated with streptavidin and packaged in a laminar flow hood by a drying agent. Store at 2-8°C.
- **E. Serum Diluent**
  - One (1) vial of serum diluent containing buffer salts and a dye. Store at 2-8°C.
- **F. Stop Solution**
  - One (1) vial of 20ml/vial - Icon L
  - One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.
- **G. Signal Reagent B – 7.0ml/vial - Icon C**
  - One (1) vial containing hydrogen peroxide (H₂O₂) in buffer. Store at 2-8°C. (See Reagent Preparation Section)
- **H. Signal Reagent A – 7.0ml/vial - Icon A**
  - One (1) vial containing hydrogen peroxide (H₂O₂) in buffer. Store at 2-8°C. (See Reagent Preparation Section)
- **I. Product Insert**

#### Procedure:

1. **Note:** Do not use reagents beyond the kit expiration date.

2. Before proceeding with the assay, bring all serum reference calibrators and controls to room temperature (20-27°C).

3. **Test procedure should be performed by a skilled individual or trained professional**

   1. **Format the microplates’ wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate format and place any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

   2. **Dispense 0.025 ml (25µl) of the appropriate serum reference calibrator, control or diluent patient specimen into the assigned well for IgG determination. For IgM or IgA, pipette 0.050 ml (50µl) of the appropriate serum reference calibrator, control or diluent patient specimen into the assigned well.

   3. **Add 0.100 ml (100µl) of H-Pylori Biotin Reagent B in A-C wells.**

   4. **Swirl the microplate gently for 20-30 seconds to mix and cover.**

   5. **Incubate 45 minutes at room temperature.**

   6. **Discard the contents of the microplate by decantation or gentle siphoning and wash as per instruction in the manufacturer’s instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoid bubble formation).**

   7. **Wash and repeat (4) additional times for a total of five (5) washes. An automatic or manual plate washer can be used. Follow the manufacturer’s instruction for proper usage. If a squeeze bottle is used, fill each well by depressing the container (avoid bubble formation).**

   8. **Always add reagents in the same order and in the same order to minimize reaction time differences between wells.**

   9. **Add 3.0 PRINCIPLE**

   10. **Add 1.0 CALCULATION OF RESULTS**

11. **Calculate the absorbance at 490nm using the microplate luminometer for at least 0.2 seconds/well.**

   12. **Read the RLUs (Relative Light Units) in each well in a microplate luminometer for at least 0.2 seconds. All results can be read within 30 minutes of adding the signal reagent.**

   **Note:** For re-assaying specimens with concentrations greater than 100 U/ml, divide the sample by a factor of 1.5 or 1.0 using the original diluted material in the serum diluent. Multiply by the dilution factor to obtain the concentration of the specimen.

13. **Note:** Do not use reagents that are contaminated or have bacterial growth.

### 9.0 TEST PROCEDURE

1. **Prepare the microplates’ wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate format and place any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2. **Dispense 0.025 ml (25µl) of the appropriate serum reference calibrator, control or diluent patient specimen into the assigned well for IgG determination. For IgM or IgA, pipette 0.050 ml (50µl) of the appropriate serum reference calibrator, control or diluent patient specimen into the assigned well.

3. **Add 0.100 ml (100µl) of H-Pylori Biotin Reagent B in A-C wells.**

4. **Swirl the microplate gently for 20-30 seconds to mix and cover.**

5. **Incubate 45 minutes at room temperature.**

6. **Discard the contents of the microplate by decantation or gentle siphoning and wash as per instruction in the manufacturer’s instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoid bubble formation).**

7. **Wash and repeat (4) additional times for a total of five (5) washes. An automatic or manual plate washer can be used. Follow the manufacturer’s instruction for proper usage. If a squeeze bottle is used, fill each well by depressing the container (avoid bubble formation).**

8. **Always add reagents in the same order and in the same order to minimize reaction time differences between wells.**

9. **Add 3.0 PRINCIPLE**

10. **Add 1.0 CALCULATION OF RESULTS**

11. **Calculate the absorbance at 490nm using the microplate luminometer for at least 0.2 seconds/well.**

12. **Read the RLUs (Relative Light Units) in each well in a microplate luminometer for at least 0.2 seconds. All results can be read within 30 minutes of adding the signal reagent.**

   **Note:** For re-assaying specimens with concentrations greater than 100 U/ml, divide the sample by a factor of 1.5 or 1.0 using the original diluted material in the serum diluent. Multiply by the dilution factor to obtain the concentration of the specimen.
2. Plot the RLUs for each duplicate serum reference versus the corresponding anti-H. Pylori activity in Units on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the level of anti-H. Pylori activity for an unknown, locate the average RLUs for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in U/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated).
5. The dose response curve (80%; 50% & 20% intercepts) should meet the following criteria should be met:
   - In order for the assay results to be considered valid the Q.C. PARAMETERS
   - Upon request from Monobind Inc.
   - The data presented in Example 1 and Figure 1 is for illustration purposes only and should not be used in lieu of a dose response curve prepared with each assay. In addition, the RLUs of the calibrators have been normalized to 100,000 RLUs for the E calibrator (greatest light output). This conversion eliminates efficiency due to variations in the efficiency of various instruments that can be used to measure light output.

### EXAMPLE 1 (Typical results for IgG, IgM or A)

<table>
<thead>
<tr>
<th>Sample</th>
<th>I.D.</th>
<th>Well</th>
<th>RLU (A)</th>
<th>Mean RLU</th>
<th>Limiting Value</th>
<th>Value (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal A</td>
<td>A1</td>
<td>1427</td>
<td>1479</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>1529</td>
<td>1298</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal B</td>
<td>D1</td>
<td>11171</td>
<td>11231</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal C</td>
<td>E1</td>
<td>26284</td>
<td>26282</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal D</td>
<td>H1</td>
<td>49362</td>
<td>51570</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal E</td>
<td>A2</td>
<td>99593</td>
<td>100000</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>100597</td>
<td>100000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>C2</td>
<td>7857</td>
<td>7756</td>
<td>6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient</td>
<td>A3</td>
<td>71874</td>
<td>70400</td>
<td>69.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The data presented in Example 1 and Figure 1 is for illustration purposes only and should not be used in lieu of a dose response curve prepared with each assay. In addition, the RLUs of the calibrators have been normalized to 100,000 RLUs for the E calibrator (greatest light output). This conversion eliminates efficiencies due to variations in the efficiencies of various instruments that can be used to measure light output.

### 1.0. Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:
1. The Dose Response Curve (80%; 50% & 20% intercepts) should be established.
2. Four out of six quality control pools should be within the established ranges.

### 12.0 RISK ANALYSIS

The MSDS and Risk Analysis Form for this product is available upon request from Monobind Inc.

12.1 Assay Performance
1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. Highly Igepamic, hemolyzed or grossly contaminated specimen(s) should not be used.
4. If more than (1) plate is used, it is recommended to repeat the dose response curve.

11.0 C.Q. PARAMETERS

### 14.0 PERFORMANCE CHARACTERISTICS

#### 14.1 Precision
The within and between assay precision of the H.Pylori AccuLite® CLIA Test System were determined by analysing on two different levels of pool control sera. The number, mean value, standard deviation (σ) and coefficient of variation for each of these control sera are presented below.

#### 14.1.1 Precision Anti-H. pylori - IgG

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>20</td>
<td>4.6</td>
<td>0.23</td>
<td>4.8%</td>
</tr>
<tr>
<td>Positive</td>
<td>20</td>
<td>46.4</td>
<td>4.6</td>
<td>6.1%</td>
</tr>
</tbody>
</table>

#### 14.1.2 Precision Anti-H. pylori - IgM

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>20</td>
<td>5.2</td>
<td>0.43</td>
<td>8.2%</td>
</tr>
<tr>
<td>Positive</td>
<td>20</td>
<td>52.5</td>
<td>4.6</td>
<td>8.4%</td>
</tr>
</tbody>
</table>

#### 14.1.3 Precision Anti-H. pylori - IgA

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>20</td>
<td>1.8</td>
<td>0.19</td>
<td>0.9%</td>
</tr>
<tr>
<td>Positive</td>
<td>20</td>
<td>44.2</td>
<td>2.35</td>
<td>5.3%</td>
</tr>
</tbody>
</table>

#### 14.1.4 Sensitivity
The sensitivity (detection limit) was ascertained by determining the variability of the '0 U/ml' calibrator and using the 2 (95% certainty) statistic to calculate the minimum dose:

<table>
<thead>
<tr>
<th>Sample</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>20</td>
<td>0.19</td>
<td>0.9%</td>
</tr>
<tr>
<td>Positive</td>
<td>20</td>
<td>44.2</td>
<td>2.35</td>
</tr>
</tbody>
</table>

### 14.2 Specificity & Accuracy
The specificity and accuracy for the Monoplate AccuLite® CLIA system were determined using the following definitions on a population of diseased and normal patients. The total number of specimens was 245.

<table>
<thead>
<tr>
<th>SPECIFICITY</th>
<th>ACCURACY</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>99%</td>
</tr>
<tr>
<td>IgM</td>
<td>91%</td>
</tr>
<tr>
<td>IgA</td>
<td>93%</td>
</tr>
</tbody>
</table>

### 15.0 REFERENCES