3.0 PRINCIPLE

Immunoenzymometric sequential assay (TYPE 4):
The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (anti-TfR and immuno- immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization time of each step during the assay is relatively short, allowing rapid saturation of the microplate wells through the interaction of streptavidin-coated on the well and excessively added biotinylated monoclonal anti-TfR antibody.

Upon mixing monoclonal biotinylated antibody, and a serum containing the monoclonal antibody, a reaction results between the native antigen and the antibody, forming an antibody-antigen complex. The interaction is illustrated by the following equation:

\[ \frac{\text{Ab}_{\text{TfR}} \cdot \text{Ag}_{\text{Antibody}} \cdot \text{B} \cdot \text{E} \cdot \text{IC} \cdot \text{Ag}_{\text{Antibody}}}{\text{IC}} \]

A suitable incubation period, the antibody-antigen bound fraction is separated from unbound antigen by decantation or aspiration. Another antibody (directed at a different epitope) labeled with an enzyme is added. Another interaction occurs to form an enzyme labeled antibody-antigen-biotinylated antibody complex on the surface of the wells. Enzyme excess is washed off a wash step. The complex is added to produce color measurable with the use of a microplate spectrophotometer. The enzyme activity on the well is directly proportional to the native antigen content.

In cases of low streptavidin serum concentrations, the use of enzyme-labeled antibodies may be considered. The detection of low concentrations of the antigen of interest relies on the high affinity and specificity of the antibodies used for the immunoassay. The detection limit of this assay is about 1-2 ng/mL.

4.0 REAGENTS

MATERIALS PROVIDED:
A. STR Calibrators - 0.5 mL  - Ions A-F
Six (6) vials of serum reference for STR at concentrations of 0 (A), 0.5 (B), 10 (C), 20 (D), 40 (E) and 80 (F) in mmol/L. A preservative has been added. Store at 2-8°C.

B. STR Enzyme Reagent  - 12.0 mL  
One (1) vial of STR (Anatrace) horseradish peroxidase (HRP) conjugate in a protein-stabilizing matrix with red dye. Store at 2-8°C.

C. STR Biotin Reagent  - 12.0 mL  - Ions A-F
One (1) vial of reagent contains anti-STR biotinylated purified rabbit IgG conjugate in buffer, blue dye and preservative. Store at 2-8°C.

D. Streptavidin Coated Plate  - 96 wells  - Ions A-F
96-well microwell coated with 1.0 µg/ml streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. Wash Solution Concentrate  - 25.0 mL
One (1) vial contains surfactant in buffered saline. An illustration is included. Store at 2-8°C.

F. Substrate Solution  - 14.0 mL  - Ions A-F
One (1) vial contains tetramethylenediamine (TMB) and hydrogen peroxide (H₂O₂) in buffer. Store at 2-8°C.

G. Stop Solution  - 8.0 mL  - Ions A-F
One (1) vial contains a strong acid (0.5M H₂SO₄). Store at 2-8°C.

H. Product Instructions.

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

Note 2: Avoid extended exposure to heat and light. Opened reagents should be used within 24 hours. Samples should be stored at 2-8°C. Kit and component stability are identified on label.

Note 3: Above reagents are for a single 96-well microplate.

4.1 Required But Not Provided:
1. Pipette (0.010 & 0.050ml) (10 & 50 μl).
2. Wash solutions (see Reagent Preparation).
3. Microplate washer or a squeeze bottle (optional).
4. Absorbent Paper for blotting the microplate wells.
5. Microtiter trays (optional).
7. Vacuum aspirator (optional) for wash steps.
8. Wash buffer ( optional).

5.0 PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

All products that contain human serum have been found to be non-reactive for Hepatitis B Surface Antigen, HIV 1&2 and HCV Antibodies by FDA required tests. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, "Biologicals in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS Publication No. 88-331, Revised 1991.

Safe Disposal of Kit components must be according to local regulatory and statutory requirement.

6.0 SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum or heparinised plasma in type, and taken with the usual precautions in the collection of venipuncture samples. For accurate comparison to establish normal values, a fasting morning serum sample should be obtained from the patient. Store the sample in a refrigerated tube with or without additives or anti-coagulants (for serum) or ethylenediaminetetraacetic acid (EDTA) or heparin (for plasma). Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

In patients receiving therapy with high biotin doses (i.e. >5mg/day), one of the specimens should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at -20°C up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.020ml (20µl) of the specimen is required.

7.0 QUALITY CONTROL

Each laboratory should assure controls at levels in the low, normal and high range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance, and high range for monitoring assay performance. These controls should be used to determine the reason for the variations.

8.0 REAGENT PREPARATION

1. Wash Buffer

Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable container. Duly buffered can be stored at 2-3°C for up to 60 days.

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

9.0 TEST PROCEDURE

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

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

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

2. Pipette 0.010ml (10µl) of the appropriate serum reference calibrator, control or specimen into the assigned well.

3. Add 0.100ml (100µl) of the STR Biotin Reagent to all wells.

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

5. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

6. Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) times for a total of three (3) washes. When a manual plate washer can be used. Follow the manufacturer’s instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Discard the excess.

7. Add 0.100ml (100µl) of Anti-STR Enzyme Reagent to all wells.

8. Cover and incubate for 30 minutes at room temperature.

9. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

11. Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate.

12. Repeat the wash steps again for a total of three (3) washes. When an automatic plate washer can be used. Follow the manufacturer’s instruction for proper usage. If a squeezed bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Discard the excess.

13. Add 0.100 ml (100µl) of substrate solution to all wells. Always add reagents in the same order to minimize reaction time differences between wells.

**DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION**

14. Incubate at room temperature for fifteen (15) minutes.

15. Add 0.050ml (50µl) of stop solution to each well and gently mix for 5 minutes. Add 0.100ml (100µl) of diluent to all wells. Incubate at room temperature. Read the absorbance in each well at 450nm using a reference wavelength, usually 660nm or 540nm depending on the instrument used. Read up to fifteen (15) minutes of adding the stop solution.

Note: Dilute the samples suspected of concentrations higher than 80nmol/L with STR’O nmol/L calibrator and multiply result by dilution factor.

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of the calibrators and patient specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.

2. Plot the absorbance for each duplicate serum reference versus the corresponding sTfR concentration in nmol/L on linear graph paper (do not average the duplicates of the serum reference calibrators)

3. Connect the points with a best-fit curve.
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) min to avoid assay drift.

3. Highly Ipecac, hemolysed or grossly contaminated specimens should not be used.

4. If more than one plate is used, it is recommended to repeat the dose response curve.

5. The addition of substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time-deviation during reaction.

6. Plate readers measure vertically, do not touch the bottom of the wells.

7. Failure to remove adhering solution adequately in the aspiration or decantation step(s) may result in poor replication and spurious results.

8. Use components from the same lot. No intermixing of reagents from different batches.

9. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Monobind IFU may yield inaccurate results.

10. All applicable national standards and regulations, including but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.

11. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.

12. Risk Analysis - as required by CE Mark IVD Directive 98/79/EC - should be strictly followed to ensure compliance and proper device usage.

12.1 Interpretation

1. Measurements and interpretation of results must be performed by a skilled individual or trained professional.

2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.

3. The reagents for the test system have been formulated to eliminate maximal interference; however, potential interaction between rare serum specimens and test reagents can cause erroneous results. Heterophilic antibodies often cause these interactions and have been known to be problems for all kinds of immunoassays (Boscato, L. M., & Stuart, M. C. Heterophilic antibodies: a problem with all immunoassays? Clin. Chem. 1998, 34-273-33). For diagnostic purposes, the results from this assay should be in combination with clinical examination, patient history and all other clinical findings.

4. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.

5. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability.

6. If computer-controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.

13.0 EXPECTED RANGES OF VALUES

In agreement with established reference intervals for a "normal" adult population, the expected ranges for the sTfR AccuBind® ELISA Test System are detailed in Table 1.

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>20</td>
<td>10.67</td>
<td>0.62</td>
<td>5.8</td>
</tr>
<tr>
<td>Normal</td>
<td>20</td>
<td>21.25</td>
<td>0.93</td>
<td>4.4</td>
</tr>
<tr>
<td>High</td>
<td>20</td>
<td>34.54</td>
<td>1.40</td>
<td>4.1</td>
</tr>
</tbody>
</table>

14.2 Sensitivity

The sTfR AccuBind® ELISA Test System has a sensitivity of 0.055 nmol/L. The sensitivity was ascertained by determining the variability of the 0 nmol/L serum calibrator and using the 2% (95% certainty) statistic to calculate the minimum dose.

14.3 Specificity

The % cross-reactivity of the sTfR antibody to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of sTfR needed to displace the same amount of labeled antibody.

---

### Table 3

Between Assay Precision (Values in nmol/L)

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>5</td>
<td>11.19</td>
<td>0.85</td>
<td>7.6</td>
</tr>
<tr>
<td>Normal</td>
<td>5</td>
<td>22.07</td>
<td>2.25</td>
<td>10.2</td>
</tr>
<tr>
<td>High</td>
<td>5</td>
<td>32.47</td>
<td>2.03</td>
<td>6.3</td>
</tr>
</tbody>
</table>

---

### Table 4

<table>
<thead>
<tr>
<th>Substance</th>
<th>Cross Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Laminin</td>
<td>ND</td>
</tr>
<tr>
<td>Human Apoferritin</td>
<td>ND</td>
</tr>
<tr>
<td>Human Heart Ferritin</td>
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</tr>
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<td>Human Spleen Ferritin</td>
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<tr>
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<td>ND</td>
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<tr>
<td>Human Serum Albumin</td>
<td>ND</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>ND</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>ND</td>
</tr>
</tbody>
</table>

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15.0 REFERENCES


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Revision: 2 Date: 2019-Jul-16 DCO: 1353
MP8625 Product Code: 8625-300

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**Note:** Computer data reduction software designed for ELISA assay may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

**EXAMPLE 1**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Well Number</th>
<th>Abs (A)</th>
<th>Mean Abs (B)</th>
<th>Value (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal A</td>
<td>A1</td>
<td>0.012</td>
<td>0.013</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>B1</td>
<td>0.013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal B</td>
<td>C1</td>
<td>0.155</td>
<td>0.156</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>D1</td>
<td>0.155</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal C</td>
<td>E1</td>
<td>0.577</td>
<td>0.567</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>0.555</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal D</td>
<td>G1</td>
<td>1.075</td>
<td>1.048</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td>H1</td>
<td>2.069</td>
<td>2.032</td>
<td>40.0</td>
</tr>
<tr>
<td>Cal E</td>
<td>I1</td>
<td>3.204</td>
<td>3.204</td>
<td>80.0</td>
</tr>
<tr>
<td></td>
<td>J1</td>
<td>3.168</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cal F</td>
<td>K1</td>
<td>0.958</td>
<td>0.978</td>
<td>18.0</td>
</tr>
<tr>
<td></td>
<td>L1</td>
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**Figure 1**

sTfR Values in nmol/L

High

Normal

Low

**TABLE 2**

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