

## **RELISA® ENA MULTIPARAMETER ANTIBODY SCREENING TEST**

### **Sm RNP SSA/Ro SSB/La Scl-70 Jo-1**

**For in vitro Diagnostic Use**

**For Professional Use**

**Catalog numbers: 7096-09 (96 wells) and 7696-09 (576 wells)**

*INTENDED USE: This is an enzyme immunoassay (EIA) test system for the detection of antibodies to the extractable nuclear antigens Sm (Smith), RNP, SSA/Ro, SSB/La, Scl-70, and Jo-1 in human serum. The results from this assay can be used as an aid in the diagnosis of autoimmune diseases.*

### **SUMMARY AND EXPLANATION OF THE TEST**

Antibodies to extractable nuclear antigens (ENA) have been associated with several autoimmune syndromes, and appear to be of diagnostic and/or prognostic significance in systemic sclerosis (1, 2), mixed connective tissue disease (3-5), Sjögren's syndrome (6, 7), polymyositis (8), dermatomyositis (9), systemic lupus erythematosus (5), and rheumatoid arthritis (10). The antinuclear antibody (ANA) test has been used as a screen for these antibodies, but the ANA does not indicate the specificity of the antibody, and antibodies to some ENA do not show a positive ANA test (11, 12). Thus, secondary confirmatory testing for antibodies to ENA is highly recommended (13).

The Sm (Smith) antigen was identified in 1966 by Tan and Kunkel as a saline soluble, non-histone glycoprotein, which is not dependent on DNA or RNA for its antigenicity (14). Antibodies to the Sm antigen are considered a specific serologic marker due to their high degree of specificity for systemic lupus erythematosus (SLE). These antibodies are seen in up to 30% of SLE patients, and have been associated with active renal disease and cerebritis (15-17).

Antibodies to Sm antigen are frequently found in conjunction with antibodies to U1-RNP in the sera of patients with SLE (18, 19). In contrast to antibodies to Sm antigen, antibodies to RNP are not considered a specific serologic marker because they are found in patients with a variety of rheumatic diseases including SLE, scleroderma, Sjögren's syndrome, and rheumatoid arthritis. However, high levels of antibody to RNP are highly associated with an overlap syndrome called mixed connective tissue disease (MCTD). Patients with MCTD are characterized by a combination of clinical features seen in SLE, scleroderma, and polymyositis. These patients frequently demonstrate a good response to corticosteroid treatment and have a lower incidence of renal disease when compared to patients with SLE (20, 21).

SSA and SSB were originally described as nuclear RNA-protein antigens in patients with Sjögren's syndrome (6, 7). Ro and La were described as cytoplasmic RNA-protein antigens in patients with SLE (22, 23). It is now widely accepted that SSA and Ro are analogous, SSB and La are analogous, and these antigens are found in both the nucleus and cytoplasm. Antibodies to SSA/Ro alone or SSA/Ro and SSB/La are found in up to 62% of patients with subacute cutaneous lupus (24), and in 85% of patients with Sjögren's syndrome who develop vasculitis (25). Antibodies to SSA/Ro alone occur in patients who have a homozygous deficiency of the C2 complement fraction (26), in primary biliary cirrhosis patients who develop Sjögren's syndrome (27), and in up to two thirds of patients with "ANA negative SLE" (28). The SSA/Ro autoantigen is a complex of the Ro60 protein and the Ro52 protein with small ribonucleoproteins. This complex is sometimes called the "SSA/Ro hY-RNA complex", and also includes the SSB/La protein. Ro60 is strongly associated with the SSA/Ro hY-RNA complex, but Ro52 is only weakly associated with the complex (29).

The Scl-70 antigen has been identified as a cellular enzyme, DNA topoisomerase I (30). Antibodies to Scl-70 have been reported in up to 56% of patients with progressive systemic sclerosis (PSS), particularly the subset of patients with diffuse scleroderma (31). These autoantibodies are considered a marker for PSS, since they are not seen in other connective tissue diseases.

Antibodies to Jo-1, which is the cellular enzyme histidyl tRNA synthetase, are found in 25-30% of patients with polymyositis or dermatomyositis, but not in other myopathies (11, 32). Anti Jo-1 antibodies have also been shown to have a high association with interstitial lung disease seen in conjunction with myositis (32).

## PRINCIPLE OF THE TEST

This test is a qualitative indirect EIA. Stabilized preparations of affinity-purified extractable nuclear antigens have been coated onto the surface of the microwells to serve as an antigenic substrate in this system. Dilutions of the patient samples are placed in the microwells and incubated, allowing specific antibodies in the sample to react with the antigen on the solid phase. After washing to remove unbound antibody and other serum proteins, the wells are incubated with goat anti-human antibodies that are labeled with horseradish peroxidase. The horseradish peroxidase-conjugated antibody preparation that is included in the test system is specific for human IgG heavy and light chains.

After incubation with horseradish peroxidase conjugate, if results are positive, a stable three-part complex is formed. This complex consists of horseradish peroxidase-conjugated anti-human antibody bound to human anti-ENA antibody, which is bound to the antigen stabilized on the plastic surface.

After another washing step, this complex is detected by adding a solution of tetramethylbenzidine (TMB) and H<sub>2</sub>O<sub>2</sub> as a chromogenic substrate. The degree of color development in each well is proportional to the concentration of anti-ENA antibodies in each serum sample. Each microwell is read in a spectrophotometer at 450 nm.

## SYSTEM COMPONENTS - MATERIALS PROVIDED

**Storage:** All components should be stored under refrigeration between 2-10°C. Do not freeze.



**Stability:** All components remain stable at least 12 months from date of manufacture. Do not use any component beyond its expiration date.



### REACTIVE REAGENTS

**Extractable nuclear antigen coated microwell strips** **PLATE**: Catalog No. 7008-09. A microwell frame containing twelve eight well strips coated with stabilized solutions of affinity purified extractable nuclear antigens. One eight well strip is used for each control or patient sample. Unused strips can be returned to the foil pouch with the desiccant pack, sealed with the zipper seal, and refrigerated for up to 45 days.

**Sample Diluent** **SOLN|DIL**: Catalog No. 7015 (14 ml). Proprietary buffered sample diluent used to dilute patient samples.

**Enzyme Antibody Reagent - Human IgG heavy and light chain specific** **CONJ|HRP**: Catalog No. 7009-09 (14 ml). Anti-human IgG (H&L) conjugated to horseradish peroxidase (HRP). Reagent is ready to use.

**Substrate Solution** **SOLN|SUB**   : Catalog No. 7035 (14 ml). HRP-specific enzyme substrate solution, containing stabilized 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Reagent is ready to use. **DANGER:** Flammable. This reagent contains less than 25% of methanol and acetone. Keep out of the reach of children. In case of contact with eyes, flush immediately and thoroughly with water and consult a physician.

**Stopping Reagent** **SOLN|STOP**   : Catalog No. 7033 (14 ml). Proprietary stopping reagent for Immuno Concepts EIA test systems. Reagent is ready to use. **DANGER:** Corrosive. This reagent contains hydrochloric and sulfuric acids (less than 3% each, by volume), and should be handled with care. Keep out of the reach of children. In case of contact with eyes, flush immediately and thoroughly with water and consult a physician. Never add water to this reagent.

**Multiparameter ENA Positive Control** **CONTROL|+**: Catalog No. 7021-09 (2 ml). Human positive control serum that contains antibodies to Sm, RNP, SSA/Ro, SSB/La, Scl-70, and Jo-1 antigens. This serum is at working dilution and is ready to use.

**ENA Negative Control** **CONTROL|-**: Catalog No. 7031 (2 ml). Human negative control serum that does not contain antibodies to Sm, RNP, SSA/Ro, SSB/La, Scl-70, or Jo-1. This serum is at working dilution and is ready to use.

### NON-REACTIVE COMPONENTS

**Holder for microwells**

**Wash Buffer Solution:**

**PBS Buffer** **PWDR|PBS**: Catalog No. 1011. Phosphate-buffered saline powder (0.01 M, pH 7.4 ± 0.2). Each pouch contains sufficient buffer powder to make one liter. (Two pouches of buffer powder are supplied for each 96-microwell plate in complete test kits).

**Wash Buffer Concentrate** **SOLN|WASH**: Catalog No. 1031 (10 ml). 5% Tween 20 solution to be used in the wash buffer. (Two vials of buffer concentrate are supplied for each 96-microwell plate in complete test kits).

**Preparation:** Dissolve one pouch of buffer powder in one liter of deionized or distilled water. Add the entire contents of one bottle of Wash Buffer Concentrate to the dissolved PBS. Mix well and store between 2-25°C for up to 4 weeks or until signs of contamination or other visible changes occur. Wash buffer solution must be at room temperature (18-25°C) before use.

## ADDITIONAL MATERIALS REQUIRED - BUT NOT PROVIDED

Volumetric precision pipettors to deliver 25-1000 µl volumes  
Squeeze bottle for delivering wash buffer solution to microwells, or an automated or semi-automated wash system for microwells  
One-liter container for PBS wash buffer solution  
Deionized or distilled water  
Plate reading spectrophotometer capable of reading absorbance at 450 nm  
Test tubes to prepare serum dilutions  
Bibulous paper or paper towels  
Multichannel pipettor capable of delivering to 8 wells  
Disposable gloves  
Lab timer

## PRECAUTIONS

1. All human source materials used for this product have been tested and found negative (not repeatedly reactive) for antibodies to Human immunodeficiency virus-1 (HIV-1), Human immunodeficiency virus-2 (HIV-2), hepatitis C virus (HCV), and for hepatitis B surface antigen (HBsAg) by FDA approved methods. However, no test method can offer complete assurance that HIV-1, HIV-2, hepatitis C, hepatitis B, or other infectious agents are absent. Thus, all kit materials should be handled in the same manner as potentially infectious materials.
2. All patient samples should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health Manual: *Biosafety in Microbiological and Biomedical Laboratories, 1999 Edition*.
3. Dilution of the components or substitution of components other than those provided in this system may yield inconsistent results.
4. Sodium azide (0.09%) is used as a preservative in the control sera. Sodium azide may react with lead or copper plumbing and form highly explosive metal azides. When disposing of reagents, flush with ample volumes of tap water to prevent potential residues in plumbing. Sodium azide is a poison and may be toxic if ingested.
5. This kit is for *in vitro* diagnostic use.
6. Never pipette by mouth and avoid contact of reagents and specimens with skin and mucous membranes. If contact occurs, wash with a germicidal soap and copious amounts of water.
7. Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.
8. Avoid splashing or generation of aerosols at all times.
9. Incubation times and temperatures other than those specified may give erroneous results.
10. Cross contamination of reagents or samples may give false results. Samples must remain confined to microwells during testing.
11. Reusable glassware must be washed and thoroughly rinsed free of detergents prior to use. All glassware must be clean and dry before use.
12. Bring all reagents, microwells, and specimens to room temperature (18-25°C) prior to use.
13. Wear disposable gloves when handling specimens and reagents, and wash hands thoroughly afterwards.
14. Microbial contamination of reagents or samples may give false results.
15. The stopping reagent is corrosive, and may cause burns. This reagent contains hydrochloric and sulfuric acids (less than 3% each, by volume), and should be handled with care. Keep out of the reach of children. In case of contact with eyes, flush immediately and thoroughly with water and consult a physician. Never add water to this reagent.

## SPECIMEN COLLECTION

**Collection:** Serum is the preferred specimen. Approximately 5 ml of whole blood should be collected aseptically by venipuncture using a sterile vacuum collection tube or other suitable collection system.

Allow blood to clot at room temperature (18-25°C). Serum should be separated from the clot by centrifugation as soon as possible to minimize hemolysis.

**Interfering Substances:** Sera exhibiting a high degree of hemolysis, icterus, lipemia, or microbial growth should not be used because these conditions may cause aberrant results. Specimens containing visible particulate matter should be clarified by centrifugation before testing.

**Storage:** Sera may be stored at 2-10°C up to one week. If testing is further delayed, sera should be stored frozen at -20°C or lower. Serum should not be stored in a self-defrosting refrigerator or freezer.

**CAUTION:** Repeated freeze/thawing of patient samples may yield false positive or false negative results.

## GENERAL PROCEDURAL NOTES

1. It is extremely important to have all kit components and serum samples at room temperature (18-25°C) before use. A full liter of wash buffer may require several hours to warm to 20°C after removal from the refrigerator. Incubation temperatures above or below the stated range may cause inaccurate results. Return unused samples and reagents to refrigerated storage after use.
2. Mix reagents well before use by gentle inversion. Do not vortex or shake reagents. Avoid foaming.
3. When preparing sample dilutions, pipette tips should be wiped prior to dispensing serum into specimen diluent. Excess sample adhering to the outside of the pipette tip will affect results.
4. The use of a multichannel pipettor is recommended because it provides more uniform reagent dispensing, incubation times, and reaction times.
5. **Adequate washing of wells is extremely important.** Inadequately washed wells will exhibit high background values, and may show false positive values. For manual washing, aspirate the contents of the wells, then fill each well with wash buffer solution. Avoid cross-contamination of the wells, particularly in the first wash after aspiration. Drain all of the wash buffer from the wells by inverting, then shaking residual wash buffer from the wells with a sharp "snapping" motion of the wrist. Repeat the filling and draining steps for a total of 3 to 5 washes. The wells should then be rapped vigorously on a paper towel or other absorbent material to remove all traces of residual wash buffer. The use of an automated microwell washing system will assure consistent washing of the wells, and is recommended.  
**NOTE:** Due to the various types of wash techniques and automated systems, the number of washes may be adjusted to obtain optimal results. Each laboratory should determine the most efficient number of washes for their washing system.
6. Inadequate removal of residual wash buffer can cause inconsistent color development. Microwell strips should be blotted on absorbent paper or towels to minimize residual wash buffer.
7. Timing of all steps is critical. All serum samples should be diluted before beginning the procedure, and they must be dispensed into the microwells in as short a period of time as possible (not more than five minutes). Batch sizes should be set so that specimen handling can be accomplished comfortably within this time period. The use of a multichannel pipettor facilitates the handling of samples and reagents, and is recommended.
8. With the exception of the last incubation (substrate solution), the start of each incubation period begins with completion of sample or reagent dispensing. The substrate solution incubation must be exactly 30 minutes for each well. All samples and reagents should be dispensed in the same sequence and at a constant rate.

## INTERPRETATION OF RESULTS

### QUALITY CONTROL

1. The blank control well should have an absorbance value of less than 0.250. Blank absorbance values greater than 0.250 indicate inadequate washing, or contamination of reagents.
2. The absorbance reading of the blank control well (row A) is subtracted from the absorbance readings of the RELISA<sup>®</sup> Procedure Check (RPC) well (row B) and each of the antigen wells (rows C through H). Net values less than zero are considered to be zero values.
3. The RELISA<sup>®</sup> Procedure Check (RPC) well (row B) should have a net absorbance greater than 0.300. Absorbance values less than 0.300 indicate inadequate color development, and an invalid run. Inadequate color development is usually due to the use of cold reagents or incorrect timing of one or more steps of the assay. Allow reagents to warm to room temperature (18-25°C), and repeat the run with particular attention paid to the timing of all steps.
4. Net absorbance values are each multiplied by 100 to obtain the value for each antigen in ENA Units.
5. The positive control serum is a pool of human serum that contains antibodies to all six autoantigens in this test. Each antigen well should show a value of 30 ENA Units or more.
6. The negative control serum is a pool of human serum that does not contain antibodies to any of the six autoantigens in this test. Each antigen well should show a value of less than 20 ENA Units.
7. Each laboratory should establish the frequency for running positive and negative control sera, based on the frequency of patient tests and the laboratory's experience with this assay.

## INTERPRETATION OF PATIENT RESULTS

1. This is a qualitative assay. The levels of antibody detected have no known clinical significance and the Unit values obtained in this assay are designed merely to separate patients into the following three broad groups. Patient sample wells that have calculated values greater than 30 ENA Units are considered to be positive. Patient sample wells that have calculated values less than 20 ENA Units are considered to be negative. Values between 20 Units and 30 Units are considered to be borderline positive and should be repeated. Each laboratory must establish its own reference range and cut-off values based on the population of patients that are being tested. Unit values are affected by patient factors, mechanical considerations (such as pipetting precision and accuracy), and assay conditions (such as temperature and timing of steps.)
2. The Sm and Sm/RNP wells are used together to determine the presence of these two autoantibodies. If the Sm/RNP well is positive, and the Sm well is negative, the patient has antibodies to RNP. If both wells are positive, with approximately equal values, the patient has antibodies to Sm. If both wells are positive, and the Sm/RNP well is 30 ENA Units or more higher than the Sm well, it is indicative of the presence of both Sm and RNP autoantibodies. If both the Sm and Sm/RNP wells are at or near their maximum absorbance, there may be a loss of discrimination between the Sm well and the Sm/RNP well. In this case, the presence or absence of RNP antibodies can no longer be accurately determined by subtracting the Sm value from the Sm/RNP value. The presence of both antibodies can be confirmed using Immuno Concepts AUTO-ID<sup>®</sup> immunodiffusion system, catalog number 6030.
3. The other wells are coated with affinity purified monospecific antigens and react only with the homologous antibody. However, patient sera with multiple antibody specificities are commonly seen in SLE and other autoimmune syndromes, so an individual serum may show a positive reaction in more than one well.
4. The wells are coated with affinity-purified antigens in the following order, from top (well A, and the solid tab end of the strip) to bottom (well H, and the notched tab end of the strip):

**Well A** – Blank control  
**Well B** – RELISA<sup>®</sup> Procedure Check (RPC)  
**Well C** – Sm  
**Well D** – Sm/RNP  
**Well E** – SSA/Ro (Ro60 and Ro52)  
**Well F** – SSB/La  
**Well G** – Scl-70  
**Well H** – Jo-1

## REPORTING OF RESULTS

Results should be reported as positive or negative for the corresponding antibodies to extractable nuclear antigens. The levels of antibodies have no known clinical significance.

## EXPECTED VALUES

The incidence of autoantibodies to various nuclear antigens varies depending upon the patient population, and the incidence of clinical rheumatic diseases in that population. The association of the antibodies with specific rheumatic diseases is summarized in Table 1.

**Table 1**

<b>Antibodies to:</b>	<b>Disease association:</b>
Sm	Highly specific marker antibody seen in 25-30% of SLE patients
U1-RNP	Mixed Connective Tissue Disease >95%; SLE 35%; lower frequency in discoid lupus or progressive systemic sclerosis
SSA/Ro	Sjögren's Syndrome 60-70%; SLE 50%
SSB/La	Sjögren's Syndrome 40-50%; SLE 15%
Scl-70	Highly specific marker antibody seen in 15-20% of PSS patients
Jo-1	Highly specific marker antibody seen in 25-30% of patients with polymyositis or dermatomyositis

## REFERENCE RANGE

The reference range was established by testing sera from 206 healthy blood donors, 105 females and 101 males, none of whom had any known history of rheumatic diseases. Additionally, data were obtained from 143 rheumatic disease patients who had antibodies to one or more of the antigens in this assay, but were negative for antibodies to the other antigens. Based on these data, the normal cut-off values were established as less than 20 ENA Units. Good laboratory practice dictates that each laboratory must establish its own normal ranges based on its patient population and other local factors.

## LIMITATIONS OF THE TEST

1. Diagnosis cannot be made on the basis of antibodies to extractable nuclear antigens alone. The physician must interpret these results in conjunction with the patient's history and symptoms, the physical findings, and other diagnostic procedures.
2. Treatment should not be initiated on the sole basis of a positive test for antibodies to extractable nuclear antigens. Clinical indications, other laboratory findings, and the physician's clinical impression must be considered before any treatment is initiated.
3. Some patients with autoimmune diseases may have undetectable or insignificant levels of antibodies to extractable nuclear antigens, and some individuals may have high levels of antibodies to extractable nuclear antigens, but little or no evidence of clinical disease. The physician must interpret the results of tests for antibodies to extractable nuclear antigens in conjunction with the patient's history and symptoms, the physical findings, and other diagnostic procedures.
4. The levels of antibody detected with this test system do not necessarily indicate severity or duration of disease.

## PERFORMANCE CHARACTERISTICS

The Immuno Concepts RELISA<sup>®</sup> Screening Assay was compared to other similar ELISA assays in commercial distribution; to double immunodiffusion and counterimmunoelectrophoresis tests done in reference laboratories; and to immunoblotting (Western Blot) results obtained with an in-house method. The results of all methods and the clinical diagnosis of the patient were considered in determining the expected or "correct" results for each sample tested. Based on these comparisons, the data in Table 2 were obtained.

**Table 2**

Antibodies to:	Relative Sensitivity	Relative Specificity	Overall Agreement
Sm	97.0%	95.5%	95.8%
Sm/RNP	94.8%	94.1%	94.4%
SSA/Ro	100%	83.3%	96.5%
SSB/La	100%	95.9%	97.9%
Scl-70	100%	100%	100%
Jo-1	100%	100%	100%

The discrepancies in specificity are attributed to the increased sensitivity of ELISA assays in comparison to "traditional" methods such as counterimmunoelectrophoresis and immunodiffusion.

### CROSSREACTIVITY

Seven samples were used for crossreactivity studies. These samples were well characterized by Western Blot, CIE, and immunodiffusion as monospecific sera for each of the autoantibodies in the RELISA<sup>®</sup> Screening Test. No cross-reactivity was noted in any of these samples. See Table 3.

**Table 3**

Sample	Antigen					
	Sm	Sm/RNP	SSA/Ro	SSB/La	Scl-70	Jo-1
Anti Sm	POS	POS	NEG	NEG	NEG	NEG
Anti RNP	NEG	POS	NEG	NEG	NEG	NEG
Anti Sm/RNP	POS	POS	NEG	NEG	NEG	NEG
Anti SSA/Ro	NEG	NEG	POS	NEG	NEG	NEG
Anti SSB/La	NEG	NEG	NEG	POS	NEG	NEG
Anti Scl-70	NEG	NEG	NEG	NEG	POS	NEG
Anti Jo-1	NEG	NEG	NEG	NEG	NEG	POS

### REPRODUCIBILITY

For each of the six antigen specificities, six samples were run on three different lot numbers of antigen strips, on three different occasions. Two of the samples were negative, but close to the 20 ENA Unit cut-off value; two samples were positive, but close to the 20 ENA Unit cut-off value; and two samples were clearly positive above the 30 ENA Unit level. In no case did a negative sample show positive results; all of the "borderline" positives consistently gave results between 20 and 30 ENA Units; and the clearly positive samples consistently gave clearly positive results.

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In the event of damage to the protective packaging, please contact Immuno Concepts prior to use.



Manufacturer



Authorized Representative in the European Community



Temperature Limitation



Contains Sufficient for <n> tests



Consult Instructions for Use



In Vitro Diagnostic Medical Device



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# RELISA® ENA TEST PROCEDURE

All samples, reagents (including the wash buffer solution), and microwells must be at room temperature before use.

- 1. PREPARE WORKSHEET**  
Label the worksheet that is enclosed in the kit to indicate the identification of each eight well strip of microwells.
- 2. PREPARE WASH BUFFER SOLUTION (PBS-Tween)**  
Dissolve contents of one PBS buffer pouch in one liter of deionized or distilled water. Add the entire contents of one bottle of Wash Buffer Concentrate to the one-liter container of dissolved PBS. Mix well. The wash buffer solution may be covered and stored at 2-25°C up to four weeks.
- 3. DILUTE PATIENT SAMPLES**  
Dilute patient samples 1:40 by adding 25 µl of serum to 975 µl of Sample Diluent. Mix well. The controls are provided at the working dilution and do not require any further dilution.
- 4. PREPARE MICROWELL STRIPS**  
Remove the required number of microwell strips from the pouch and place them in the frame holder. The microwells must be firmly seated in the frame holder. Press down firmly on both ends of the strips so that they securely snap into the frame holder. Wells that are properly seated in the frame holder will not fall out when the frame holder is inverted. Unused strips can be returned to the foil pouch, sealed with the zipper seal, and refrigerated for up to 45 days.
- 5. DISPENSE SERUM DILUTIONS**  
Dispense 100 µl of diluted patient sample into each of the eight wells of one multiparameter strip.
- 6. INCUBATE STRIPS (30 minutes at room temperature, i.e., 18-25°C)**  
Incubate at room temperature for 30 minutes. The wells should be protected from drafts or shifts in temperature during incubation. If desired, the wells can be covered with transparent tape or a paper towel to protect them from dust or other foreign bodies.
- 7. WASH STRIPS (See General Procedural Notes 5 and 6)**  
Wash the wells 3 to 5 times with PBS-Tween Wash Buffer Solution. For manual washing, aspirate the contents of the wells, then fill each well with wash buffer solution. Avoid cross-contamination of the wells, particularly in the first wash after aspiration. Drain all of the wash buffer from the wells by inverting, then shaking residual wash buffer from the wells with a sharp “snapping” motion of the wrist. Repeat the filling and draining steps for a total of 3 to 5 washes. The wells should then be rapped vigorously on a paper towel or other absorbent material to remove all traces of residual wash buffer.
- 8. DISPENSE ENZYME ANTIBODY REAGENT**  
Dispense 100 µl of Enzyme Antibody Reagent into each of the wells.
- 9. INCUBATE STRIPS (30 minutes at room temperature, i.e., 18-25°C)**  
Incubate at room temperature for 30 minutes. The strips should be protected from drafts or shifts in temperature during incubation. If desired, the strips can be covered with transparent tape or a paper towel to protect them from dust or other foreign bodies.
- 10. WASH STRIPS**  
Wash the wells 3 to 5 times with PBS-Tween Wash Buffer Solution. For manual washing, aspirate the contents of the wells, then fill each well with wash buffer solution. Avoid cross-contamination of the wells, particularly in the first wash after aspiration. Drain all of the wash buffer from the wells by inverting, then shaking residual wash buffer from the wells with a sharp “snapping” motion of the wrist. Repeat the filling and draining steps for a total of 3 to 5 washes. The wells should then be rapped vigorously on a paper towel or other absorbent material to remove all traces of residual wash buffer.
- 11. DISPENSE SUBSTRATE SOLUTION**  
Using a timer to assure consistent intervals, dispense 100 µl of Substrate Solution to each of the wells. The Substrate Solution must be added to the wells at a steady rate, so that each well is incubated for exactly the same length of time (30 minutes). The substrate solution in wells incubated with positive samples will turn blue, and the solution in wells incubated with negative samples will be colorless to very pale blue.
- 12. INCUBATE STRIPS (Exactly 30 minutes at room temperature, i.e., 18-25°C)**  
Incubate at room temperature for exactly 30 minutes. The strips should be protected from drafts or shifts in temperature during incubation.
- 13. DISPENSE STOPPING REAGENT**  
After the first well has incubated for exactly 30 minutes, add 100 µl of Stopping Reagent to each well, in the same order and at the same rate that the Substrate Solution was added to the wells. Upon addition of stopping reagent, blue substrate solution will turn yellow and colorless solution will remain colorless.
- 14. READ ABSORBANCE OF WELLS**  
Within 30 minutes after addition of the Stopping Reagent, the wells must be read in a plate reading spectrophotometer. The wells are read at 450 nm against the blank control well. If a dual wavelength spectrophotometer is available, the wavelength for the reference filter should be set at 600-650 nm. Reading the microwells without a reference filter will result in higher absorbance values.

#### FOR TECHNICAL ASSISTANCE:

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