RELISA® MPO-ANCA ANTIBODY TEST SYSTEM

For in vitro Diagnostic Use
For Professional Use
Catalog number: 7096-15

INTENDED USE: This is an enzyme immunoassay test system for the detection of antibodies to myeloperoxidase (MPO) in human serum. This test system is to be used as an aid in the detection of antibodies associated with microscopic polyangiitis, idiopathic necrotizing and crescentic glomerulonephritis, and other vasculitides.

Summary and Explanation of the Test

Antineutrophil cytoplasmic autoantibodies (ANCA) are a group of antibodies that react with cytoplasmic antigens in human neutrophils. Although these antibodies were originally reported in 1964 (1), the first report linking these antibodies to disease was in 1982, when Davies et al. reported the antibodies in eight patients with segmental necrotizing glomerulonephritis (2). In 1984, four more patients with vasculitis and glomerulonephritis were reported. In 1985, van der Woude et al showed that ANCA had a high association with granulomatosis with polyangiitis, and that antibody titer correlated with disease activity (3). In 1988, Falk and Jennette reported that ANCA have more than one antigen specificity (4). A subsequent report showed that the specificity of ANCA correlated with the pathologic features of vasculitides (5).

Screening for ANCA is usually performed using an indirect immunofluorescent assay. In this test, several patterns of cellular staining may be seen. Two major patterns of staining have been described and well characterized when ethanol-fixed neutrophils are used in the immunofluorescent ANCA test. Autoantibodies that show a fine granular cytoplasmic pattern, called C-ANCA, are usually directed against a serine protease, Proteinase 3 (PR-3). These autoantibodies have been shown to have a high association with granulomatosis with polyangiitis. The other major pattern of staining, the perinuclear, or P-ANCA pattern, which is usually due to antibodies directed against myeloperoxidase (MPO), has been associated with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis (4). All samples that show a positive indirect immunofluorescent test should be confirmed as MPO-ANCA or PR3-ANCA using an enzyme immunoassay (EIA). Some authors feel that all samples from clinically suspected patients should be tested by EIA, since 5% of samples are positive only by EIA (6). Although PR3-ANCA are seen most commonly in patients with granulomatosis with polyangiitis (up to 85% of patients), they are not specific for this disease, since they can also be seen in a smaller percentage of patients with idiopathic necrotizing and crescentic glomerulonephritis. Similarly, MPO-ANCA are associated with idiopathic necrotizing crescentic glomerulonephritis in up to 65% of patients, but may also be seen in a smaller percentage of granulomatosis with polyangiitis patients. Either MPO-ANCA or PR3-ANCA can be seen in microscopic polyangiitis, each in approximately 45% of patients (7, 8, 9).

Principle of the Test

This test is a semi-quantitative indirect EIA. Stabilized human MPO 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 gamma chains.

After incubation with horseradish peroxidase conjugate, a stable three part complex is formed if results are positive. This complex consists of horseradish peroxidase-conjugated anti-human antibody bound to human antibodies to MPO, which are 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_2O_2 as a chromogenic substrate. The degree of color development in each well is proportional to the concentration of antibodies to MPO 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

RELISA® MPO coated microwell strips PLATE: Catalog No. 7008-15. A microwell frame containing twelve eight well strips coated with human MPO. These strips are color coded gray. If fewer than eight wells are needed for testing, the wells can be separated by snapping them apart. The 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.

RELISA® Sample Diluent SOLN|DIL: Catalog number 7100 (100 ml). Proprietary buffered sample diluent used to dilute patient samples.

RELISA® Enzyme Antibody Reagent - Human IgG gamma chain specific CONJ|HRP: Catalog number 7009-15 (14 ml). Anti-human IgG (gamma chain specific) conjugated to horseradish peroxidase (HRP). Reagent is ready to use.

RELISA® Substrate Solution SOLN|SUB  : Catalog number 7035 (14 ml). HRP-specific enzyme substrate solution, containing stabilized 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2). 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.

RELISA® 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.

RELISA® MPO Standard Sera CAL: Catalog numbers 7261-15, 7262-15, 7263-15, 7264-15, 7265-15 (2 ml each). Human sera that contain antibodies to MPO. The assay value for each of these sera is stated on the vial label. These sera are at working dilution and are ready to use.

RELISA® MPO Positive Control CONTROL| + : Catalog number 7021-15 (2 ml). Human positive control serum that contains antibodies to MPO. This serum is at working dilution and is ready to use.

RELISA® Negative Control CONTROL| - : Catalog number 7031 (2 ml). Human negative control serum that does not contain antibodies to MPO. This serum is at working dilution and is ready to use.

RELISA® Optional MPO Undiluted Assayed Positive Control OPT+: Catalog number 7022-15 (0.25 ml). Human positive control serum that contains antibodies to MPO. Treat this positive control as an undiluted serum. The assay value for this serum is stated on the vial label.

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 to be 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. Sodium azide may react with lead or copper plumbing and form explosive metal azide salts. 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 its 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 15 minutes for each well. All samples and reagents should be dispensed in the same sequence and at a constant rate.

Interpretation of Results

CALCULATIONS

1. Subtract the absorbance value of the reagent blank well from the absorbance values obtained in standard, control, and patient sample wells. Calculate the mean absorbance values for duplicate wells.

2. Plot the mean absorbance value of each standard on the RELISA® MPO Standard Set Worksheet. Draw a best fit line between the standard points.
3. Obtain the unit value of each patient sample by interpolating from the Standard Line.

OPTIONAL SINGLE POINT CALIBRATION METHOD

1. Subtract the absorbance value of the reagent blank well from the absorbance values obtained in standard, control, and patient sample wells. Calculate the mean absorbance values for duplicate wells.
2. Divide the specific antibody concentration of the #3 Standard Serum (stated on the label) by the mean absorbance value of the calibrator wells to obtain the Conversion Factor.
3. Multiply the absorbance values of each of the samples by the Conversion Factor to obtain the specific antibody concentration in units.
4. The simplified form of these calculations can be expressed as:

$$\frac{\text{\#3 Standard Value (Units)}}{\text{Absorbance of \#3 Standard}} \times \text{Absorbance of Sample*} = \text{Unit Value for Sample}$$

*If Standards and samples are run in duplicate, use the average absorbance of the duplicate wells.

QUALITY CONTROL

1. The mean absorbance value of the #3 Standard must be at least 0.400. Absorbance values less than 0.400 indicate inadequate color development, and an invalid run. Inadequate color development is usually due to 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.
2. The blank control well should have an absorbance value of less than 0.150. Blank absorbance values greater than 0.150 indicate inadequate washing or contamination of reagents, and an invalid run.
3. Samples with specific antibody values greater than the upper limit of the #5 Standard should be reported as greater than the unit value of the #5 Standard.
4. The Standard Line must be plotted for each run (or the Conversion Factor must be calculated if you are using the optional single point calibration). Using a Standard Line or Conversion Factor from another run will invalidate the results.
5. Each laboratory should establish and maintain its own reference (normal) range values, based on the patient population and other local factors.
6. The positive control serum is a human serum that contains antibodies to MPO. This is a qualitative control which should give a value of greater than 35 units.
7. The negative control serum is a pool of human serum that does not contain antibodies to MPO. This control should give values of less than 35 units.
8. The undiluted assayed positive control serum is a human serum that contains antibodies to MPO. The assay value of this control, in MPO units, is stated on the label. This range was established to encompass 99% of the values expected due to statistically normal variation. Occasional small deviations outside these ranges are expected. Each laboratory should establish its own accept/reject criteria based on its experience with this assay.

INTERPRETATION OF PATIENT RESULTS

This is a semi-quantitative assay. The levels of antibodies to MPO have been shown to rise and fall with the course of disease, but the clinical significance of a single antibody level is still under study (9). The unit values obtained in this assay are designed merely to separate patients into the following broad groups. Patient sample wells that have calculated values greater than or equal to 35 units are considered to be positive. Patient sample wells that have calculated values less than 35 units are considered to be negative. 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.) Serial determinations of antibody levels on a patient may indicate the rise or fall of antibody levels.

REPORTING OF RESULTS

Results should be reported as positive or negative for antibodies to MPO, with the unit value. The levels of antibodies found in a single sample have limited clinical significance. Serial determinations of antibody levels on a patient may indicate the rise or fall of antibody levels, which have been shown to follow disease course.

Limitations of the Test

1. Diagnosis cannot be made on the basis of MPO-ANCA antibody detection 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 MPO. Clinical indications, other laboratory findings, and the physician's clinical impression must be considered before any treatment is initiated.
3. The results of this test should be used in conjunction with information available from the clinical evaluation and other diagnostic procedures to determine the patient's clinical status.

Expected Values

In a normal population, the expected value is less than 35 units (negative). MPO-ANCA have been reported in 77% of patients with necrotizing and crescentic glomerulonephritis, 65% of patients with necrotizing vasculitis, 60% of patients with polyarteritis nodosa, 60% of patients with eosinophilic granulomatosis with polyangiitis, 45% of patients with microscopic polyangiitis, and 10% of patients with granulomatosis with polyangiitis (9).

REFERENCE RANGE
The reference range was established by testing sera from 500 healthy blood donors, 239 females and 261 males, none of whom had any known history of rheumatic diseases. Based on a Receiver Operator Curve generated from these data, the normal cut-off value was established as less than 35 MPO Units.

Because of the inherent variability of ELISA assays, Unit values within five Units above or below the positive/negative cut-off value (i.e. between 30 and 40 Units) should be interpreted with care. The clinical findings, signs, symptoms, the physician's impression, and other laboratory results should be assessed in interpreting the results of this assay.

Performance Characteristics

The Immuno Concepts RELISA® MPO Test System was compared to another ELISA MPO test system that is in commercial distribution. The population studied consisted of 177 samples which were submitted to clinical laboratories for ANCA, MPO, and PR3 testing, but without specific diagnoses, 10 samples were from patients with a diagnosis of microscopic polyangiitis, 15 samples were from patients with a diagnosis of necrotizing crescentic glomerulonephritis, 12 samples were from patients with a diagnosis of polyarteritis nodosa, 7 samples which were from patients with a diagnosis of eosinophilic granulomatosis with polyangiitis, 12 patients with a diagnosis of vasculitis, 25 patients with a diagnosis of granulomatosis with polyangiitis, 261 samples from male blood donors, and 239 samples from female blood donors. All samples were tested in parallel on the predicate device and the subject device. Based on these comparisons, the following data were obtained using the five-point calibration curve:

		Predicate anti-MPO Test	
Immuno Concepts		Positive	Negative
RELISA® MPO	Positive	53	38
Antibody Test	Negative	2	665

These data yield the following statistics: relative sensitivity, 96.4%; relative specificity, 94.6%; and overall agreement, 94.7%.

Among the 38 "false positive" samples, 28 showed a P-ANCA pattern by immunofluorescence, 3 showed a C-ANCA pattern by immunofluorescence, 3 showed an atypical ANCA pattern by immunofluorescence, and 4 did not demonstrate any immunofluorescent pattern.

The following data were obtained using the optional single point calibration method:

		Predicate anti-MPO Test	
Immuno Concepts		Positive	Negative
RELISA® MPO	Positive	53	38
Antibody Test	Negative	2	665

These data yield the following statistics: relative sensitivity, 96.4%; relative specificity, 94.6%; and overall agreement, 94.7%

REPRODUCIBILITY

The precision of the assay was measured using seven samples that had MPO-ANCA values within the range of the calibration curve. These samples were run in duplicate on three different lot numbers of antigen coated microwell strips on three different occasions by three different technologists. The intraassay and interassay precision is shown in the following Tables:

INTRA-ASSAY PRECISION

n=21	Concentration (Units)	S.D.	%C.V.
Sample 1	64	8	12
Sample 2	59	5	8
Sample 3	42	3	7
Sample 4	40	3	8
Sample 5	97	4	4
Sample 6	45	4	9
Sample 7	102	5	5

INTER-ASSAY PRECISION

n=3	Concentration (Units)	S.D.	%C.V.
Sample 1	65	9	14
Sample 2	59	5	8
Sample 3	43	3	7
Sample 4	40	4	10
Sample 5	97	9	10
Sample 6	45	8	18
Sample 7	103	5	5

LINEARITY

Within the range of the calibration curve the assay is linear , as demonstrated using the methods shown in the NCCLS Guideline, *Evaluation of the Linearity of Quantitative Analytical Methods* (10).

VALIDATION OF SINGLE POINT CALIBRATION

The use of a single point calibrator was validated using the same panel of 758 sera that were used for comparison to the predicate device. Regression analysis of this comparison showed a regression coefficient (r^2) of 99.4%, and analysis of variance (ANOVA) of the data showed that there was no statistically significant difference between the two methods From a practical point of view, in this comparison we saw only ten samples (1.3%) that showed diagnostic discrepancies between the five point calibration system and the optional single point calibration system. All of these samples had MPO Unit values close to the cutoff of 35 Units, ranging from 33 to 37 Units (10). This type of sample is a diagnostic problem in any assay system, and needs to be considered carefully by the laboratory professionals who analyze the samples and interpret the data.

CROSSREACTIVITY STUDIES

A total of 40 sera that contained autoantibodies other than anti-MPO-ANCA were tested using the Immuno Concepts RELISA® MPO Test System. These samples included the common antinuclear antibody patterns, such as homogeneous, speckled, and nucleolar, as well as antibodies directed against cytoplasmic components such as mitochondria, Golgi apparatus, and the cytoskeleton. Twenty of the samples contained rheumatoid factors. None of these samples produced a positive result in the Immuno Concepts RELISA® MPO Test System.

Bibliography

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10. Data on file, Immuno Concepts, N.A., Ltd.

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


Manufacturer

EC REP Authorized Representative in the European Community


Temperature Limitation

Contains Sufficient for <n> tests


Consult Instructions for Use

IVD In Vitro Diagnostic Medical Device

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RELISA® ANCA-MPO 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 location of the samples in the microwells. Assay the standards in duplicate. To use the multi-point curve, all five standard sera must be run. For the optional single point calibration method, run only the #3 Standard in duplicate. One well is used for a reagent blank. We recommend that each control and patient sample be assayed in duplicate until an acceptable precision for the assay has been established in your laboratory.

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. To use the optional MPO undiluted assayed positive control, dilute it in the same manner as the patient samples. Mix well. The calibrator, positive control, and negative control are provided at the working dilution and do not require any further dilution.

4. **PREPARE MICROWELLS**
   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. If using individual wells or less than a full strip of wells, be sure each well is firmly seated. Wells that are properly seated in the frame holder will not fall out when the frame holder is inverted. If fewer than eight wells are needed for testing, the wells can be separated by snapping them apart. Unused wells 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 the calibrators, controls, and diluted patient samples into the appropriate wells as outlined on the worksheet. Dispense 100 µl of Sample Diluent into the reagent blank well.

6. **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.

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 to 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 (15 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 15 minutes at room temperature, i.e., 18-25°C)
    Incubate at room temperature for exactly 15 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 15 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 at 450 nm without a reference filter will result in higher absorbance values.

FOR TECHNICAL ASSISTANCE:
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