



EL-RF/3™ (IgM-IgG-IgA)

*An enzyme immunoassay for the detection and measurement of
IgM, IgG, and IgA Isotypes of Rheumatoid Factor*

Instruction Manual

Catalog No.:
303-305

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INTRODUCTION

Name: TheraTest EL-RF/3™ (IgM-IgG-IgA)

Intended Use

FOR IN VITRO DIAGNOSTIC USE ONLY

The TheraTest EL-RF/3™ (IgM-IgG-IgA) is an in vitro diagnostic test that measures rheumatoid factor (RF) of the IgM, IgG, and IgA classes in human serum and is intended as an aid to the diagnosis of Rheumatoid Arthritis (RA).

Summary and Explanation of Test

RF describes antibodies (IgM, IgG and IgA) that are directed toward antigenic determinants present on human and animal IgG.

RF-IgM activity is detected by latex agglutination, nephelometry, radioimmunoassay (RIA) or enzyme linked immunosorbent assay (ELISA); ELISA and RIA have been proposed as the best techniques to measure RF-IgM activity.¹⁻⁵

Although primarily associated with RA, RF-IgM activity also has been identified in sera of patients with other rheumatic disorders (e.g., Sjögren's syndrome, systemic lupus erythematosus), with non-rheumatic infectious and inflammatory diseases (e.g., sarcoidosis and subacute bacterial endocarditis) and with aging. RA is a common disease that has a prevalence of approximately 1%. Consequently the diagnostic specificity (20%) and predictive value (10%) of an RF-IgM test for RA is low.⁶ The specificity and predictive value of the RF test may be increased, however, by simultaneously measuring the three RF isotypes: RF-IgA RF-IgM, and RF-IgG.⁷ When all three RF isotypes are elevated, the specificity and predictive value of RF testing improve substantially (see GUIDE TO INTERPRETATION).⁸

Method Description

The TheraTest EL-RF test system is a solid phase enzyme linked immunosorbent assay with a 96-well plate format that is designed to measure serum RF activity. Half the wells of the polystyrene plate have been coated with rabbit IgG and half serve as blank microwells, (i.e., lack antigen). Rabbit IgG has a greater specificity for RA than does human IgG.⁹ The microwells are incubated with patient Specimens, Controls, and Calibrators. During incubation, the antibody in the test sample binds to the solid phase antigen. After incubation, unbound antibody is removed by aspiration and washing.

For the measurement of RF-IgM, rabbit anti-human IgM (Fc₅μ specific) labeled with horseradish peroxidase (HRP) is added to the wells. A specific substrate is added and the presence of RF-IgM is detected by a color change that is measured with an ELISA reader. For calculation of the net absorbance value for the sample, the absorbance value for the blank microwell (i.e., Specimen Blank) is subtracted from the value obtained for the antigen-coated microwell.

For the measurement of RF-IgA, the same procedure is followed as for RF-IgM detection, except that a rabbit anti-human IgA antibody (α-chain specific), labeled with horseradish peroxidase (HRP) is substituted for the HRP-labeled anti-IgM antibody.

For the measurement of RF-IgG, the sample is first digested with pepsin. This enzyme destroys RF-IgM and a large proportion of RF-IgA activity. The resultant IgG (Fab')₂ fragments, however, are able to react with antigen-coated wells and binding is detected with a rabbit anti-human IgG (Fab')₂ specific antibody coupled to HRP.

REAGENTS

EL-RF/3™ (IgM-IgG-IgA) Kit (Cat. No. 303-305), sufficient for 60 determinations (3 isotypes).

Upon receipt, store reagents at 2° - 8°C. Do not freeze. Allow all reagents to equilibrate to room temperature (18° - 25°C) prior to use. Return all reagents to refrigeration (2° - 8°C) immediately after use.

Kit Components

1. RF Microwell plate (Fig. 1)

Half the microwells are coated with rabbit IgG and the other half are saturated with blocking protein to serve as blank microwells (i.e., Specimen Blanks). The microwells are alternately coated on each column. The number of antigen-coated microwells in a kit is sufficient to perform 60 x 3 tests.

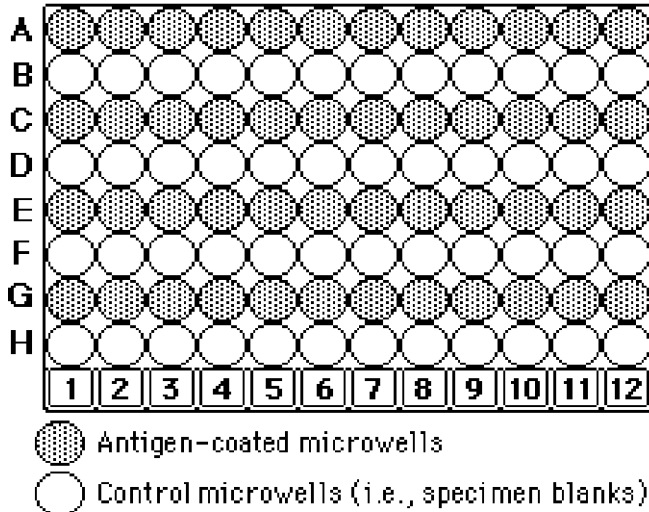


Fig 1. Distribution of antigen-coated and control microwells for EL-RF 96 microwell plate

2. **Wash Buffer (10X):** 10X concentrated buffer with Tween 20. This solution is also used as a Specimen Diluent.
3. **Anti-IgA Enzyme Conjugate-Rb:** Rabbit anti-human IgA antibody (α -chain specific) labeled with HRP (yellow solution)
4. **Anti-IgM Enzyme Conjugate-Rb:** Rabbit anti-human IgM (Fc₅ μ specific) coupled with HRP (blue solution).
5. **Anti-IgG (Fab')₂ Specific Enzyme Conjugate-Rb:** Rabbit anti-human IgG (Fab')₂ antibody labeled with HRP (green solution).
6. **Chromogen:** a ready to use solution, containing both the peroxide substrate of Horseradish Peroxidase (HRP) and tetramethylbenzidine as chromogenic indicator.
7. **Stop Reagent:** 2M phosphoric acid.
8. **RF-IgA Calibrator*:** Human serum containing RF-IgA in buffer with preservative.
9. **RF-IgA Positive Control*:** Human serum containing RF-IgA in buffer with preservative.
10. **RF-IgM Calibrator*:** Human serum containing RF-IgM in buffer with preservative.
11. **RF-IgM Positive Control*:** Human serum containing RF-IgM in buffer with preservative.
12. **RF-IgG Calibrator*:** Human serum containing RF-IgG in buffer with preservative.
13. **RF-IgG Positive Control*:** Human serum containing RF-IgG in buffer with preservative.
14. **Negative Control*:** Human serum with preservative.
15. **Pepsin Concentrate:** Pepsin in a stabilizing solution.

16. Pepsin Buffer: Buffer for the dilution of pepsin concentrate and digestion of serum.

17. Pepsin Stop Solution: Buffer with Tween 20.

18. Color-Coded Card for Strips: A color-coded card for a 96 well plate, with a frame printed on it, to guide the addition of the three antibody conjugates: anti-IgM Enzyme Conjugate-Rb (blue), anti-IgA Enzyme Conjugate-Rb (yellow), and anti-IgG (Fab')₂ Specific Enzyme Conjugate-Rb (green).

19. Sponge: A sponge to absorb any spills.

* Reagents containing sodium azide

WARNINGS AND PRECAUTIONS

FOR IN VITRO DIAGNOSTIC USE ONLY

Reagents Containing Human Source Material

CAUTION: Controls and Calibrators contain Human Serum. Treat as potentially infectious.

The materials used to prepare the Calibrator and Controls were derived from human blood. When tested by FDA-cleared methods for the presence of antibody to HIV (Human Immunodeficiency Virus) and Hepatitis B Surface antigen (HBsAg), the materials were nonreactive. Inasmuch as no test method can offer complete assurance that HIV, hepatitis virus or other infectious agents are absent, these materials and all patient specimens should be handled as though capable of transmitting infectious diseases. Human material should be handled in accordance with good laboratory practice using appropriate precautions as described in the Centers for Disease Control and Prevention/National Institutes of Health Manual, "Biosafety in Microbiological and Biomedical Laboratories", 4th edition, 1999. HHS Publication (NIH and CDC). Web site: <http://bmbi.od.nih.gov/>

Reagents Containing Sodium Azide

Calibrators and Controls contain sodium azide which can react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush drain with large quantities of water to prevent azide build-up.

Hazardous Substance Risk & Safety Phrases:

R22 - Harmful if swallowed.

R36/37/38 - Irritating to eyes, respiratory system, and skin. Avoid inhalation and direct contact.

S26 - In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

S28 - After contact with skin, wash immediately with plenty of water.

S36/37/39 - Wear suitable protective clothing, gloves and eye/face protection

S46 - If swallowed, seek medical advice immediately and show this container label

Stop Reagent (2M Phosphoric Acid)

May cause severe burns upon contact with skin. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. On contact, flush with copious amounts of water for at least 15 minutes.

European Community Hazardous Substance Risk and Safety Phrases (Council Directive 88/379/EEC).

R34 – Causes burns.

S26 – In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

S36/37/39 – Wear suitable protective clothing, gloves and eye/face protection.

S45 – In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).

Chromogen

Irritant! This product contains 3,3',5,5'-tetramethylbenzidine (TMB) ($\leq 0.05\%$), a chromogenic indicator of horseradish peroxidase activity. It has shown neither mutagenic nor carcinogenic effects in laboratory experiments (28).

Hazardous Substance Risk & Safety Phrases:

R36/37/38 – Irritating to eyes, respiratory system, and skin. Avoid inhalation and direct contact.

S24/25 – Avoid contact with skin or eyes.

S26 – In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

S36 – Wear suitable protective clothing.

S51 – Use only in well-ventilated areas.

General Precautions and Information

1. Do not use components after expiration date.
2. Do not mix reagents from different lots.
3. Use gloves while handling specimens and kit reagents; wash hands thoroughly after completion of tests.
4. Do not pipette by mouth.
5. Do not eat, drink, or smoke in work areas.
6. Avoid microbial contamination of reagents. If solutions become turbid, they should not be used.
7. Avoid exposure of reagents to excessive heat or light during storage.
8. Chromogen contains an organic solvent that irritates eyes and mucous membranes.
9. Do not allow Chromogen to come in contact with metals or oxidizing agents.
10. Use disposable glassware and plasticware or wash all labware thoroughly according to standard laboratory practice.

11. Ensure that the Pepsin Stop solution is not interchanged with the Stop Reagent.

12. Check for any crystals in the Pepsin Buffer, Pepsin Stop Solution, and Wash Buffer (10X) prior to use; redissolve if necessary.

SPECIMEN REQUIREMENTS

No special preparation of the patient is required. A whole blood specimen should be obtained (in a red top tube) using acceptable medical techniques to avoid hemolysis. The blood should be allowed to clot and the serum separated by centrifugation. Unseparated blood can be stored at 18° - 25°C for 24 hours before serum separation. The test serum should be clear and non-hemolyzed. Serum samples may be stored at 2° - 8°C for up to 14 days prior to testing. After 14 days, serum samples must be re-drawn. Warm the thawed serum sample to room temperature prior to testing. Do not use serum that has been thawed more than once. **DO NOT FREEZE** unseparated blood. If the diagnosis of Cryoglobulinemia is considered, a false negative test is minimized by clotting the blood at 37°C followed by immediate testing of the isolated serum.

PROCEDURE

A. Materials Provided (60 tests)

Table 1.	Item	Quantity
1.	EL-RF 96-Microwell Plates	5
2.	10X Wash Concentrate-TW	2 x 100 mL
3.	Anti-IgM Enzyme Conjugate-Rb	2 x 18 mL
4.	Anti-IgA Enzyme Conjugate-Rb	30 mL
5.	Anti-IgG (Fab') ₂ Enzyme Conjugate-Rb	20 mL
6.	Chromogen	2 x 27 mL
7.	Stop Reagent	2 x 27 mL
8.	RF-IgM Calibrator	0.35 mL
9.	RF-IgM Positive Control	0.35 mL
10.	RF-IgA Calibrator	0.35 mL
11.	RF-IgA Positive Control	0.35 mL
12.	RF-IgG Calibrator	0.1 mL
13.	RF-IgG Positive Control	0.1 mL
14.	Negative Control	0.35 mL
15.	Pepsin Concentrate	2 x 1.0 mL
16.	Pepsin Buffer	60 mL
17.	Pepsin Stop Solution	60 mL
19.	Color-coded Frame for Strips 1	
20.	Sponge 1	

B. Materials Required but not Provided

- 96-well format rack with 1.2-2 mL minitubes
- Strips of caps to fit the minitubes
- Precision micropipettors that deliver 5 μ L and 100 μ L
- Multichannel (8/12) pipettor that delivers 100 μ L and 200 μ L
- Disposable plastic pipette tips
- Reagent grade deionized water (e.g., CAP Type 1)
- Timer
- Pipettes (5 mL, and 10 mL)
- Multichannel pipette reagent reservoirs
- Single or dual wavelength ELISA reader (with 450 nm filter) for 96-well microtiter plates
- Incubator or water bath capable of maintaining a temperature of 37°C.
- Optional: Multichannel Repipettor (50-200 μ L) or semi-automatic plate washer.

C. Reagent Preparation for Assay

1. **Wash Buffer:** The 10X Wash Buffer-TW is diluted 1:10 prior to use (e.g., 100 mL of Wash Buffer (10X) is used to prepare 1000 mL of 1X Wash Buffer). When stored at 2° - 8°C, the 1X Wash Buffer is stable for 8 weeks. The 1X Wash Buffer is used to dilute samples (i.e., as Specimen Diluent) and to wash microwells.
2. **Chromogen:** Disposable glassware or plasticware should be used to handle the Chromogen. Alternatively, all labware employed must be washed thoroughly according to standard

laboratory practice. The Chromogen should be pipetted into the reagent reservoir (an excess of 10% of the necessary amount should be measured) no sooner than 5 minutes prior to use. Discard any unused Chromogen after completion of the test procedure. Do not pour excess Chromogen back into the stock bottle.

3. Patient Specimens, Positive Control, Negative Control, and Calibrator.

Note: The Specimens, Calibrator, and Controls for the RF-IgG assay must be prepared 2-3 hours before the assay to allow time for the pepsin digest. Preparations of Specimens, Calibrators, and Controls for the RF-IgM and RF-IgA assays may be done either at the same time or immediately before the assay. Dilution procedures should adhere to standard laboratory practice.

- a) Place minitubes without caps in rows A and E of a minitube rack as shown in **Fig. 2a** [p. 9]. Write the specified information on each minitube as follows: for row A, begin with RF-IgA Calibrator (CalA) followed by the RF-IgA Positive Control (PCA), the Negative Control (NC) and the Patient Specimens (S1, S2, S3...S9). For row E begin with RF-IgG Calibrator (CalG) followed by the RF-IgG Positive Control (PCG), the Negative Control (NC) and the patient Specimens (S1, S2, S3....S9). A single plate is sufficient to test nine patient Specimens.
- b) Place two additional minitubes in columns 1 and 2 of row C. Write the information shown in **Fig. 2a** [p. 9] on these minitubes.
- c) **Dilute the Pepsin Concentrate 1:20** (e.g., 0.5 mL of Pepsin Concentrate plus 9.5 mL of Pepsin Buffer); mix well. **Add 0.5 mL of the diluted Pepsin Solution to each minitube in row E (Fig. 2a [p. 9]).**
- d) Add 1 mL of 1X Wash Buffer to minitubes in rows A and C.
- e) Add 5 μ L of the following to their respective minitubes:
 - RF-IgA Calibrator (A1)
 - RF-IgA Positive Control (A2)
 - RF-IgM Calibrator (C1)
 - RF-IgM Positive Control (C2)
 - RF-IgG Calibrator (E1)
 - RF-IgG Positive Control (E2).
- f) Add 5 μ L of the Negative Control and patient Specimens to their corresponding minitubes in rows A and E.

- g) Cap all of the minitubes and invert them ~10 times to mix. Observe movement of air bubble to ensure proper mixing.
- h) **The sample minitubes in row E, containing the Pepsin Digest solution, are transferred to another rack which is placed in a 37°C incubator or water bath for 2-3 hours.**

D. Assay Procedure

1. Bring reagents to room temp. (18° - 25°C) for 30 minutes.
2. Arrange the plate of microwells as shown in **Fig. 2b** [p. 9]. Number the textured tabs of the strips and place the strips in the frame with these tabs downward.
3. Transfer minitubes that underwent the 37°C incubation to row E of the minitube rack.
4. Inspect the Pepsin Stop Solution for crystals. If necessary, warm at 37°C until crystals have dissolved. Add 0.5 mL of the Pepsin Stop Solution to each tube in row E and replace caps.
5. Mix all minitubes as follows:
 - a) Ensure minitubes are capped well;
 - b) Place the inverted lid of the rack on top of capped tubes;
 - c) Press firmly and invert 10 times.
 - d) Observe movement of the air bubble to ensure proper mixing.

Note: *Since the samples may be retested, the sample caps are replaced after first use and the minitubes are stored at 2-8°C. Discard the samples if final results are satisfactory.*

6. **Load Plate:** Add 100 µL of each diluted sample to the appropriate microwell. An example of efficient loading of the plate with the help of a multichannel pipettor is as follows: (see **Fig. 2b** [p.9]):
 - a) Load Calibrator and Positive Control for RF-IgM and for RF-IgA, as shown in **Fig. 2b** [p. 9], with a single- or multi-channel channel pipettor. First add RF-IgA Calibrator to microwells A1 and B1 (see **Sector I, Fig. 2a and 2b** [p. 9]). Then add RF-IgM Calibrator to microwells C1 and D1. For the Positive Controls, the operation is repeated: RF-IgA Positive Control is added to microwells A2 and B2 and RF-IgM Positive Control is added to microwells C2 and D2.
 - b) With a multichannel pipettor, transfer 100 µL from the remaining minitubes in row A (i.e., Negative Control and Patient Specimens) to

microwells in rows A, B, C and D of columns 3-12 (**Sector II, Fig 2** [p. 9]).

- c) Eject all tips and replace with new tips.
- d) Using the multichannel pipettor with tips on each channel, transfer solutions from minitubes 1-12 in row E of the holder to microwells in columns 1-12 in rows E, F, G and H (**Sector III**). All designated wells are now filled.
7. **Incubate the plate** for 30±5 min. at room temperature (18° - 25°C).
8. **For manual washing**, remove the sample fluid from the wells by aspiration or by shaking the liquid from the plate into a designated disposal container or sink. Fill all wells with 1X Wash Buffer (250-350 µL per well) but do not overflow wells. Remove liquid from wells and repeat the washing procedure 2-3 more times for a total of 3-4 washes. After the last wash, remove all residual fluid from the wells by tapping the inverted plate on absorbent paper. For washing with automated or semi-automated instruments, follow the manufacturer's accompanying instructions.
9. Three different **Enzyme Conjugates** are used. Set up three reagent reservoirs: anti-IgA (yellow), anti-IgM (blue) and anti-IgG (Fab')₂ (green). Use a multichannel pipettor or similar device to add 100 µL of Conjugate. Place the plate on the color-coded frame to help in guiding this step.
 - add anti-IgA (yellow) to rows A and B;
 - add anti-IgM (blue) to rows C, D, E and F;
 - add anti-IgG (Fab')₂ (green) to rows G and H.

Note: To verify that IgM has been digested, anti-IgM Enzyme Conjugate is added to wells containing samples incubated with pepsin. This is referred to as the IgG Digest Control.
10. **Incubate the plate for 30±5 minutes** at room temperature (18° - 25°C). Return residual Enzyme Conjugates to corresponding bottles for future use. During the last 2-4 minutes, pipette volume of Chromogen needed for **Step 12** into reagent reservoir.
11. **Wash Plate 3 times**, as described in **Step 8**. Tap inverted plate on absorbent paper.
12. **Add 100 µL of Chromogen** into all microwells.
13. **Incubate the plate for 15±1 minutes** at room temperature (18° - 25°C). Microwells with Calibrators, Positive Controls and positive patient Specimens will turn blue.

- 14. Pipette 100 µL of Stop Reagent** into all microwells and mix by gently tapping the plate. The color will change from blue to yellow.
- 15. Read Absorbance:** Firmly seat the plate in reader and measure absorbance of each microwell at 450 nm. Values should be measured within 30 minutes of assay completion. If a dual wavelength ELISA reader is used, set test wavelength at 450 nm and reference at 620-690 nm. Follow instructions provided with your instrument.

E. Procedural Notes

- **Handling of microwells** - Promptly replace unused microwells in the metallized pouch with desiccant and reseal. To avoid false positive readings, the bottom of the microwells should be kept clean at all times.
- **Crystallization of Reagents** - The Wash Buffer (10X), Pepsin Buffer, or Pepsin Stop Solution may crystallize at 2° - 8°C. Inspect each for crystals prior to use. If crystals are present, warm the reagent at 37°C until crystals dissolve.
- **Washing** - Microwells may be washed with a multichannel pipettor or a multichannel repeat-pipettor. Commercial semi-automated washing systems may be used following the manufacturer's instructions. Irrespective of the method, blot the wells on absorbent paper after the final wash. *Note: Insufficient washing may elevate background giving imprecise test results.*
- **Pipetting** - To avoid cross-contamination and sample carry-over, Calibrators, Positive Controls, Negative Controls, and patient Specimens must be pipetted with separate pipette tips. A multichannel pipettor may be used to pipette the Specimens, Enzyme Conjugates, 1X Wash Buffer, Chromogen and Stop Reagent.
- **WARNING** - The Stop Reagent is used to stop the Chromogen reaction. The Pepsin Stop Solution is used to terminate pepsin digestion. They are not the same reagent. Use each one only for its intended use.

QUALITY CONTROL

- 1. Specimen Blank:** For a Specimen Blank, the absorbance value should be <0.3. If the absorbance value for multiple Specimen Blanks exceeds this limit, it indicates that an excess of Enzyme Conjugate was added to the wells. If repeated testing demonstrates that the absorbance value for a particular Specimen Blank and antibody class is elevated, the test should be repeated at a higher serum dilution (e.g., 1:400 or 1:800) until the Blank value falls below 0.2. Elevated values for Specimen Blanks may be caused by hypergamma-globulinemia. If the Blank value is still high after repetition of the test, contact the manufacturer.
- 2. Patient Specimens:** If the net absorbance value for a Patient Specimen exceeds 2.5 OD, retest by further diluting the Specimen 1:10 (final 1:2010) and repeat the test procedure. To obtain Units/mL, use the following formula:
- $$\text{CF} \times \text{Net Abs.}_{(10\text{-fold diluted Specimen})} \times 10 = \text{Units/mL in Specimen}$$
- Note: An elevated RF-IgG value without an associated increase in RF-IgM and/or RF-IgA is very unusual. The test should be repeated.*
- 3. Calibrator:** The Calibrators should have net absorbance values that are within the ranges shown on the Data Sheet. TheraTest RF-IgM Units (I.U.) are traceable to the World Health Organization Reference Preparation for Rheumatoid Factor. International standards for RF-IgA and RF-IgG are not available.
- 4. Positive and Negative Controls:** Positive and Negative Controls should be run with each test procedure. The acceptable ranges of Unit values (Units/mL) for the Controls are listed in the Data Sheet. When results for Positive and Negative Controls are not within the specified ranges, the run is repeated. Previous runs of the same lot should be reviewed to determine if a shift or a sustained change (e.g., downward trend) in values has occurred. For the Positive Control:
- a) A sudden upward shift suggests:
 - insufficient dilution;
 - accidental contamination of well with Enzyme Conjugate.
 - b) A downward shift suggests:
 - excessive dilution;
 - quality of the water for diluted Wash Buffer is poor.
 - c) A downward trend suggests:
 - loss of antibody or antigen;
 - poor storage or transport conditions.
- When a plausible explanation for an aberrant result is not apparent, repeating the test is highly recommended.

- 5. Measurement of RF-IgG:** To ensure that pepsin has destroyed RF-IgM, the RF-IgG Calibrator, RF-IgG Positive Control, and RF-IgG Negative Control developed with anti-IgM Enzyme Conjugate-Rb (row E) should yield a low raw absorbance value (e.g., = 0.200 OD). For Patient Specimens the raw absorbance value in row E should not exceed 0.250.

A high value for row E (E1 and E2) indicates poor enzymatic digestion (consider dilution, stability, time and temperature of incubation). Low values for the wells in row C (C1 and C2) indicate poor function of the IgM Enzyme Conjugate-Rb.

If corrective action and repeated testing fails to solve the problem, contact the manufacturer.

- 6. Testing of QC Samples:** An RF-IgM International Standard (available from Centers for Disease Control and Prevention, Atlanta, Georgia), based on the WHO reference preparation, contains 1,000 I.U. of RF-IgM activity per mL. To obtain an acceptable absorbance, dilute the standard 1:2010 (final), instead of the regular 1:201, in Specimen Diluent before testing it. International Standards are not available for RF-IgA and RF-IgG.

RESULTS

A. Calculation of Results

Determination of net Absorbance values

The net absorbance value for each sample (i.e., Calibrator, Positive Control, Negative Control, and Specimens) is calculated by subtracting the absorbance value of the Blank microwell from the absorbance value of its antigen-coated microwell.

Note: Microwells in rows A, C, E and G contain antigen while microwells in B, D, F and H are their respective Specimen Blanks.

EXAMPLE:

Absorbance of Sample in coated microwell = 1.250

Absorbance of Sample in blank microwell = 0.250

Net Absorbance of Sample = 1.250 - 0.250 = 1.000

Calculating RF-IgM Units

Determine the net absorbance for RF-IgM Calibrator, Controls, and patient Specimens. The number of I.U. (International Units) / mL of RF-IgM is given on the Data Sheet and/or on the vial containing the RF-IgM Calibrator.

$$\frac{\text{I.U. / mL of RF-IgM Calibrator}}{\text{Net Abs. of RF-IgM Calibrator}} = \text{Conversion Factor (CF)}$$

$$\text{CF} \times \text{Net Abs. for Sample} = \text{I.U./mL RF-IgM Activity}$$

EXAMPLE:

Calibrator = 180 I.U. / mL

Net Absorbance of Calibrator = 1.20

Net Absorbance of Sample = 0.60

$180 \div 1.2 = 150$, then $150 \times 0.6 = 90 \text{ I.U. / mL}$

Calculating RF IgA and RF-IgG Units

To determine RF-IgA and RF-IgG activity, perform a similar set of calculations using the appropriate Calibrators' assigned Unit values. Computer controlled instrumentation may facilitate calculation of the results.

B. Limitations of the Procedure

The EL-RF-IgM test should not be performed on grossly hemolyzed, microbially contaminated, or grossly lipemic samples. Only serum has been tested for these interfering contaminants. Performance with other specimen types has not been determined.

Diagnosis should not be based solely on a positive test result. All information (patient history, physical examination and other data) is essential to diagnose RA. Furthermore, a negative result does not exclude RA.

When an RF-IgA value exceeds 1000 U/mL, the RF-IgG value may be due in part to RF-IgA activity. Therefore, the RF-IgG may be falsely elevated. However, 1000 U/mL of RF-IgA is almost exclusively limited to RA.⁸

EXPECTED VALUES

Expected net absorbance and Unit values for Calibrators and Control Sera are shown on the Data Sheet enclosed with the kit.

Normal limits have been defined based on a study of 100 blood bank donors (95% for RF-IgM and RF-IgA; 99% for RF-IgG). The upper limits of normal for RF-IgM, RF-IgG and RF-IgA are 25 I.U./mL, 20U/mL, and 35.U/mL, respectively. Three (3%) of the 100 normals tested had both RF-IgM and RF-IgA while none had all three classes of RF (see **Fig. 3**).

Among 97 RA patients, values ranged as follows: RF-IgM: 1-7400 I.U./mL, RF-IgG: 0-1,230 Units/mL, and RF-IgA: 0-6,000 Units/mL. In 71 patients with generalized inflammatory and autoimmune diseases, values ranged as follows: RF-IgM: 0-259 I.U./mL, RF-IgG: 0-91 Units/mL, and RF-IgA: 4-436 Units/mL. In 89 patients with various

rheumatic conditions, primarily osteoarthritis and fibrositis, values were: RF-IgM: 0-156 I.U./mL, RF-IgG: 2-47 Units/mL, and RF-IgA: 15-290 Units/mL (see **Fig. 3**).

GUIDE TO INTERPRETATION

Depending on the technique, elevated levels of RF activity may be detected in as many as 60-90% of RA patients. Abnormal RF activity is prevalent, however, in the elderly (10-15%) as well as in many other disorders:⁶ juvenile polyarticular RA (60-75%), Sjögren's syndrome (55-95%), cryoglobulinemia (40-100%), viral infection (15-65%), leprosy (5-60%), MCTD (15-60%), subacute bacterial endocarditis (25-50%), systemic lupus erythematosus (15-35%), silicosis (30-50%), interstitial pulmonary fibrosis (10-50%), hepatitis (15-40%), sarcoidosis (3-33%), polymyalgia rheumatica (5-10%), asbestosis (30%), and syphilis (0-15%).

Among 257 patients seen at a rheumatology clinic, the diagnoses were RA (n=97) and non-RA (n=160) giving a prevalence of RA at 38%. When tested by the EL-RF/3 (IgM-IgA-IgG), the results indicate that the specificity and predictive value of the EL-RF/3 (IgM-IgG-IgA) test was higher than for RF-IgM alone⁸ (**Table 2**). Unlike the test group, the general population shows a prevalence of approximately 1%. The predictive value of a diagnostic test is inversely related to the prevalence of the disease the test is intended to detect. Therefore, the predictive values described in **Table 2** are overestimates. This caveat aside, the diagnostic value of the EL-RF/3 (IgM-IgG-IgA) supersedes the value observed for measurement of RF-IgM isotype alone.

Table 2. Sensitivity, specificity and predictive value of EL-RF/3 (IgM-IgA-IgG) for diagnosis of RA when the disease was observed at a prevalence of 38% in a rheumatology clinic setting (n=257 patients).

RFTest	Sensitivity	Specificity	Predictive Value
IgM	90%	74%	68%
IgM+IgA	77%	87%	79%
IgM+IgG+IgA	57%	98%	96%

PERFORMANCE DATA

A. Accuracy: RF-IgM values by EL-RF/3 (IgM-IgG-IgA) test were compared to RF activity by a nephelometric method for thirty RA and thirty normals. **Table 3** shows the number of individual positive and negative tests by each method, whereas **Table 4** shows the calculated test sensitivities and specificities.

Table 3. Comparison of positive and negative results for sera tested simultaneously by EL-RF/3 (RF-IgM-IgG-IgA) kit and by a nephelometry method*.

		EL-RF/3 (IgM-IgG-IgA) kit	
		Positive	Negative
Nephelometry	Positive	27	0
	Negative	2	31

*A test was considered positive if one of the three isotopes was positive during EL-RF/3 (IgM-IgG-IgA) testing.

Table 4. Relative sensitivity and specificity of EL-RF/3 (IgM-IgG-IgA) kit and nephelometry kit for diagnosis of RA among 30 patients and 30 normals

	EL-R/3 (IgM-IgG-IgA) Kit	Nephelometry
Sensitivity	93%	87%
Specificity	97%	97%

For the 30 RA patients, results obtained by nephelometry were plotted against the isotype measured by the RF/3 (IgM-IgG-IgA) method; linear regression analysis was performed yielding a correlation coefficient of $r=0.90$ for RF-IgM (**Fig. 4**). The correlation coefficients were 0.33 and 0.62 for RF-IgG and RF-IgA, respectively.

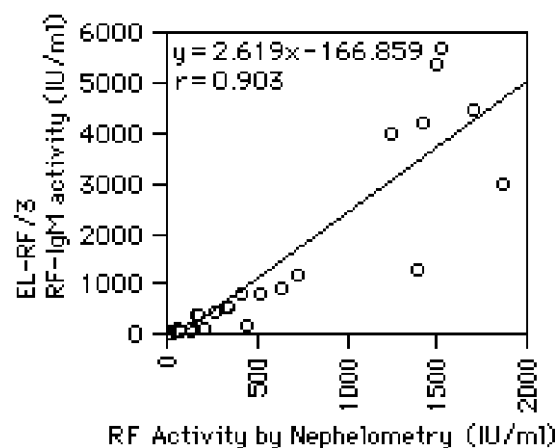


Fig. 4. Correlation between RF-IgM levels measured by EL-RF/3 (RF-IgM-IgG-IgA) kit and RF activity by a nephelometric method in 30 RA patients.

B. Precision: Within-run precision was performed with Specimens that were known to contain three different levels of RF-IgM, RF-IgG and/or RF-IgA activity. Serum samples were tested repeatedly in a single run (n = 10) yielding coefficients of variation less than 10%. Between-run precision was determined by assaying a Specimen containing RF-IgM, RF-IgG and RF-IgA in 12 separate runs (plates); coefficient of variation was less than 10%.

C. Antigenic Test Specificity: Test specificity of the TheraTest EL-RF/3 (IgM-IgG-IgA) Test System was determined by inhibiting antibody binding with a soluble form of aggregated human IgG. The Calibrators and Positive Controls were incubated with pooled human IgG and the mixture was subsequently incubated with coated wells. Over 80% of the absorbance was inhibited.

TROUBLESHOOTING

Problem	Possible Causes	Solution
Values for Calibrators and/or Controls are out of range.	<ol style="list-style-type: none"> 1. Incorrect temperature, timing, or pipetting; reagents not mixed properly. 2. Cross contamination of Controls. 3. Improper dilution. 4. Optical pathway (bottoms of wells) not clean. 5. Wavelength of filter incorrect. 6. Blank OD > value listed on Data Sheet. 	<ol style="list-style-type: none"> 1. Check that temperature was correct. Check that time was correct. See "Poor Precision" (below) No. 2-4. Repeat test. 2. Pipette carefully. 3. Repeat test. 4. Check for moisture or dirt. Wipe bottoms of wells and reread. 5. Change filter to 450 ± 5 nm. 6.a) Chromogen contaminated. Replace if solution is contaminated. b) The incubation time was too long. c) Insufficient washing. d) A damaged or dirty well.
No positive reactions on plate.	<ol style="list-style-type: none"> 1. One or more reagents not added, or added in wrong sequence. 2. Improper dilution of Wash Buffer. 3. Antigen-coated plate inactive. 	<ol style="list-style-type: none"> 1. Recheck procedure. Check for unused solutions. Repeat test. 2. Repeat test. 3. Check for obvious moisture in unused wells. Rerun test with Controls only for activity.
All wells turn yellow upon addition of Stop Reagent.	<ol style="list-style-type: none"> 1. Contaminated Chromogen. 2. Contaminated buffers and reagents. 3. Wash Buffer (1X) contaminated. 4. Improper dilution of serum. 	<ol style="list-style-type: none"> 1. Check absorbance of unused Chromogen 2. Check all solutions for turbidity. 3. Use clean container. Check quality of water used to prepare buffer. 4. Repeat test.
Poor precision.	<ol style="list-style-type: none"> 1. Pipettor delivery CV greater than 5% or samples not added slowly. 2. Serum or reagents not mixed sufficiently; reagents not at room temperature prior to addition. 3. Reagent addition taking too long; inconsistency in timing intervals, air bubbles. 4. Air currents blowing over plate during incubations. 5. Optical pathway not clean. 6. Instrument not equilibrated before readings were taken. 7. Washing not consistent; trapped bubbles; liquid left in wells at end of wash cycle. 8. Improper pipetting. 	<ol style="list-style-type: none"> 1. Check calibration of pipettor. Use reproducible technique. 2. Mix all reagents gently but thoroughly and equilibrate to room temperature. 3. Develop consistent uniform technique and avoid splashing or use multi-tip device or autodispenser to decrease time. 4. Cover plate or place it in chamber. 5. Wipe bottom of plate with soft tissue. Check instrument light source and detector for dirt. 6. Check instrument manual for warm-up procedure. 7. Use only acceptable washing devices. Lengthen timing delay on automated washing devices. Check that all wells are filled and aspirated uniformly. Dispense Wash Buffer above level of reagents previously added to wells. 8. Avoid air bubbles in pipette tips.

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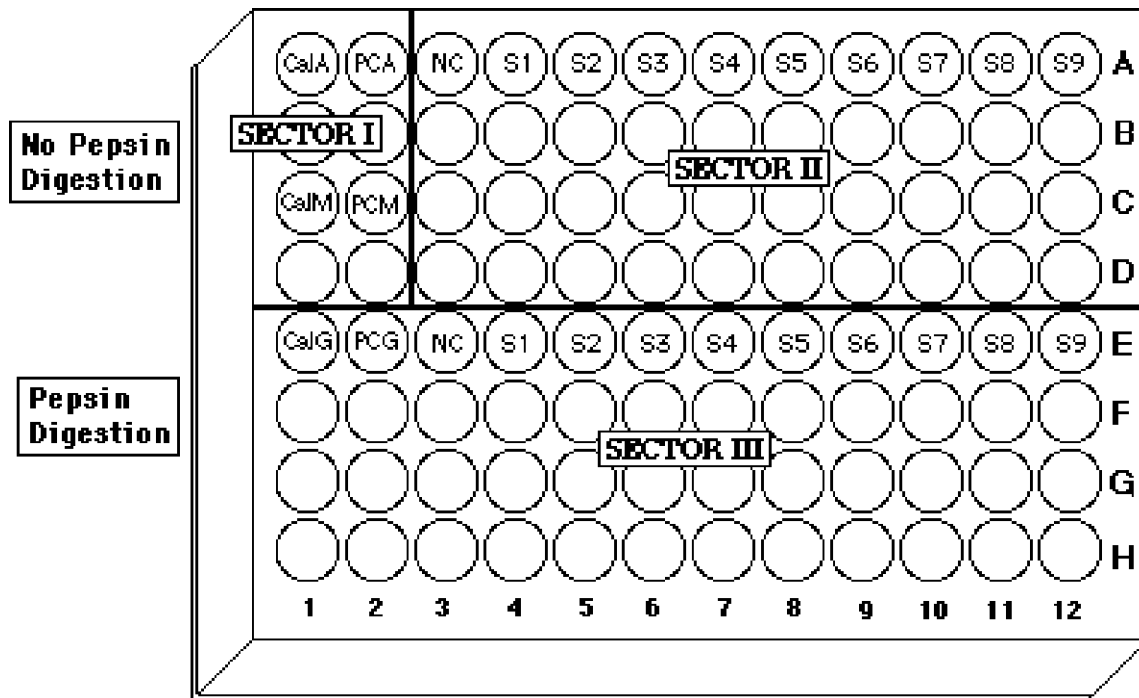


Fig. 2a. Example for configuring minitubes in rack for RF testing. (i) Using a single channel pipettor, transfer samples from minitubes in SECTOR I to microwells; (ii) Using a multichannel pipettor, transfer samples from minitubes in SECTOR II to microwells; (iii) Using a multichannel pipettor, transfer samples from minitubes in SECTOR III to microwells.

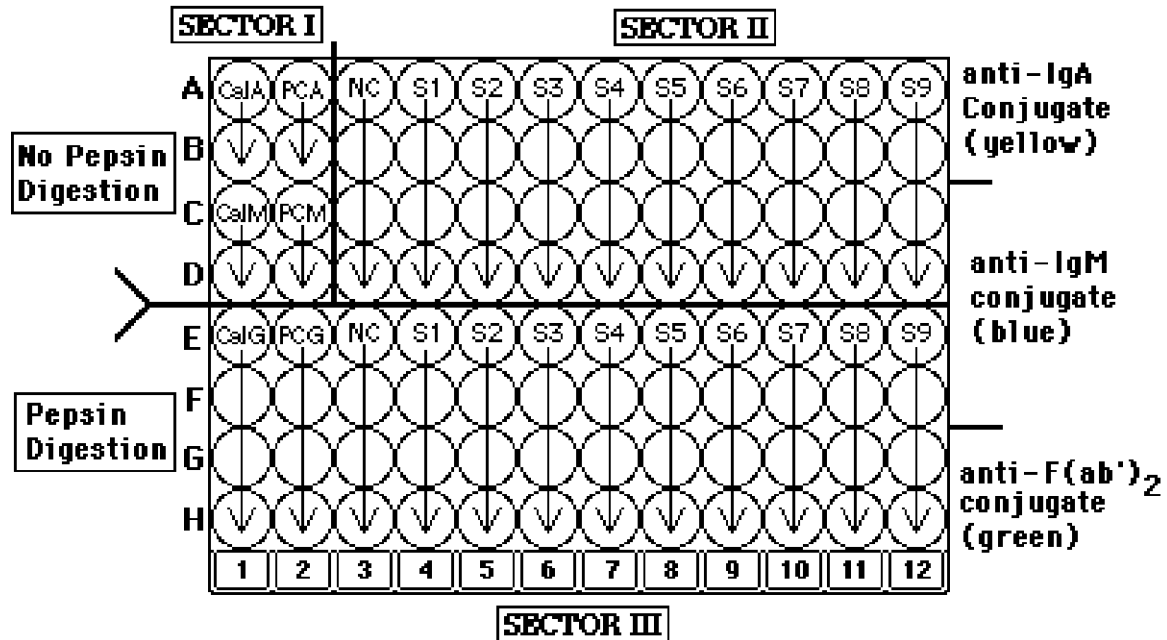


Fig. 2b. Positions for Controls and Patient Specimens on RF 96-microwell plate. SECTOR I receives samples from minitubes as shown. SECTOR II receives samples from minitubes in row A and SECTOR III receives samples from minitubes in row E. The symbol, (↓), indicates that all microwells receive the same sample.

Key for Figures

CalM: RF-IgM Calibrator	PCM: RF-IgM Pos. Control
CalA: RF-IgA Calibrator	PCA: RF-IgA Pos. Control
CalG: RF-IgG Calibrator	PCG: RF-IgG Pos. Control
S#: Patient Specimen	NC: Negative Control

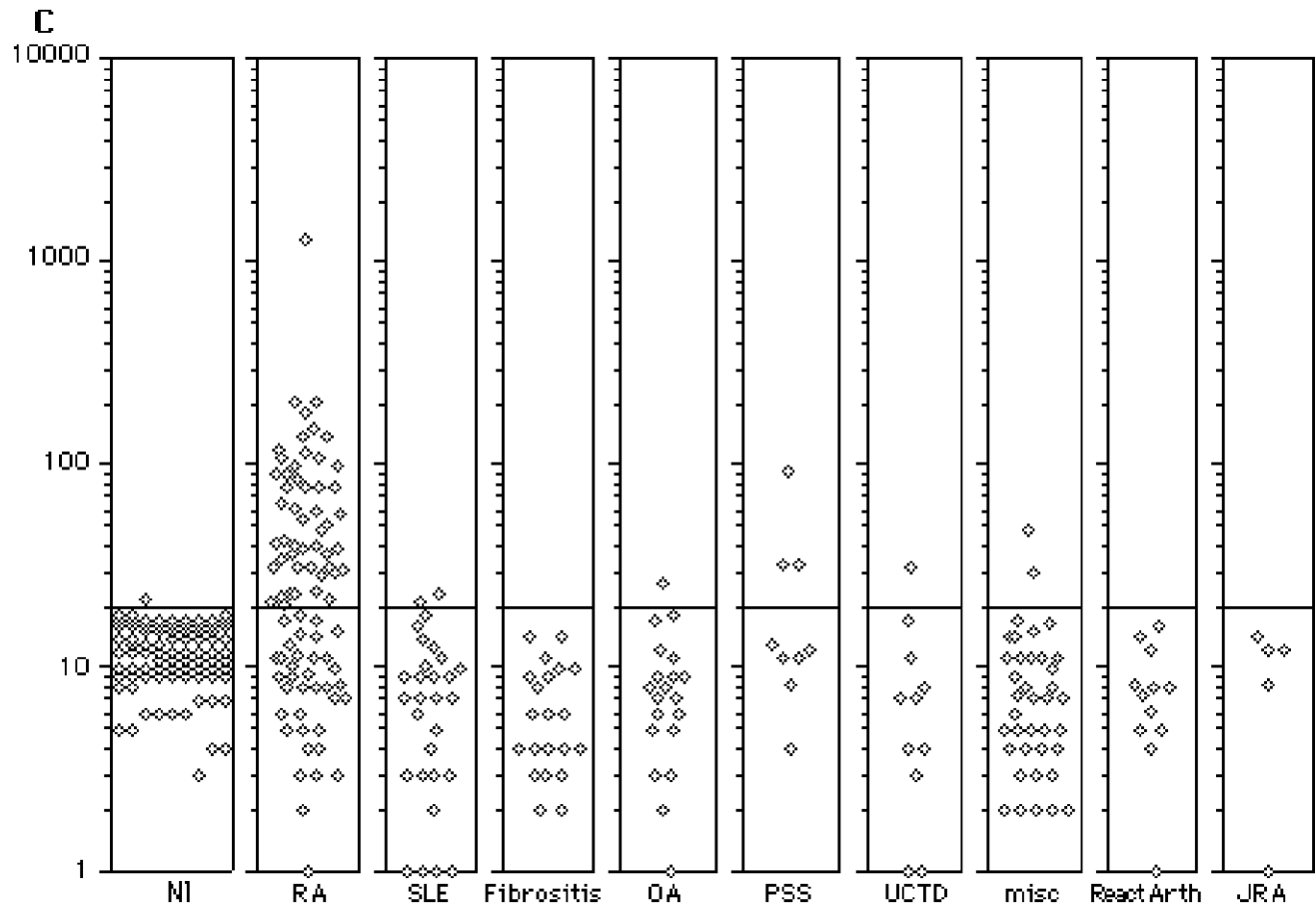


Fig. 3. Scattergrams of RF-IgM, RF-IgA, and RF-IgG levels among RA patients, Disease Controls and Normals. **Panel A:** RF-IgM levels; **Panel B:** RF-IgA Levels and **Panel C:** RF-IgG levels. **NL:** Normals (n=100); **RA:** rheumatoid arthritis (n=97); **SLE:** systemic lupus erythematosus (n=32); **Fibrositis** (n=22); **OA:** osteoarthritis (n=21); **PSS:** progressive systemic sclerosis (n= 8); **UCTD:** undifferentiated connective tissue diseases (n=12); **Misc:** miscellaneous non-inflammatory disorders (n=46); **React Arth:** reactive arthritis (n= 13) and **JRA:** juvenile rheumatoid arthritis (n=5). Note log scale and (—) that shows upper limit of normal: RF-IgM, 25 I.U./mL; RF-IgG, 20 U/ml; and RF-IgA, 35 U/ml.



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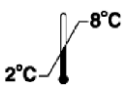
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Abbreviated Test Procedure EL-RF/3 (IgM-IgG-IgA)

- 1. Dilute Calibrators, Positive Controls, Negative Control and Specimens as required for IgG pepsin digest/assays and for IgA and IgM assays**
 - a) Dilute Pepsin Concentrate 1:20 and add 0.5 mL of the diluted Pepsin Solution to minitubes in row E.**
 - b) Add 1 mL of 1X Wash Buffer (Specimen Diluent) to minitubes in rows A and C.**
 - c) Add 5 μ L of Calibrators and Positive Controls to the appropriate dilution minitubes: CalA (A1), PCA (A2), CalM (C1), PCM (C2), CalG (E1), PCG (E2).**
 - d) Add 5 μ L of the Negative Control (A3, E3) and patient Specimens (A4-A12 & E4-E12) to appropriate minitubes in rows A and E.**
 - e) Mix all dilution tube contents well.**
- 2. Transfer sample minitubes in row E to 37°C & incubate for 2-3 hours; then return tubes to row E at room temperature (18° - 25°C).**
- 3. Stop the Pepsin Digests by adding 0.5 mL of Pepsin Stop Solution to each minitube; mix tube contents well.**
- 4. Transfer 100 μ L of diluted Calibrators and Positive Controls for IgA and IgM (Cal A, PCA, CalM, PCM) into the designated paired wells (antigen-coated and Blank wells). NO PEPSIN TREATMENT!!**
- 5. Transfer 100 μ L of diluted Negative Control and patient Specimens into the designated duplicate paired wells: one pair for RF-IgA and one pair for RF-IgM tests. NO PEPSIN TREATMENT!!**
- 6. Transfer 100 μ L of diluted Calibrator, Controls, and Specimens treated with Pepsin (row E) into the designated duplicate paired wells: one pair for the IgG Digest Control (to be developed with Anti-IgM Enzyme Conjugate) and one pair for the RF-IgG tests (to be developed with the Anti-IgG Enzyme Conjugate). PEPSIN TREATED SAMPLES!!**
- 7. Incubate the plate for 30 \pm 5 minutes at room temperature (18° - 25°C).**
- 8. Wash the wells three times with 1X Wash Buffer.**
- 9. Add 100 μ L of the appropriate Enzyme Conjugate to the designated wells. (Follow the color code displayed on the color-coded frame.)**
- 10. Incubate the plate for 30 \pm 5 minutes at room temperature (18° - 25°C).**
- 11. Wash the wells three times with 1X Wash Buffer.**
- 12. Add 100 μ L Chromogen to each well.**
- 13. Incubate plate for 15 \pm 1 minutes at room temperature (18° - 25°C).**
- 14. Add 100 μ L Stop Reagent to each well.**
- 15. Read the absorbance at 450 nm, reference 620-690 nm within 30 minutes.**