THERATEST LABS
Innovations in Immunoassays

EL-RF IgM™

An enzyme immunoassay for the detection and measurement of
IgM Rheumatoid Factor

Instruction Manual

Catalog No.: 301-305

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INTRODUCTION

Name: TheraTest **EL-RF-IgM™**

Intended Use

IVD

The TheraTest EL-RF-IgM kit is an *in vitro* diagnostic test kit that measures rheumatoid factor of the IgM class (RF-IgM) in human serum and is intended as an aid to the diagnosis of Rheumatoid Arthritis (RA).

Summary and Explanation Of Test

RF describes antibodies that are directed toantigenic 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 are relatively low.[6] When all three RF isotypes (RF-IgM, RF-IgG, & RF-IgA) are elevated, as may be detected with an RF/3 test kit, the specificity and predictive value for RA improve substantially.[7]

Method Description

The TheraTest EL-RF-IgM test system is a solid phase enzyme linked immunosorbent assay designed to measure serum RF-IgM activity. Half the microwells of the polystyrene plate have been coated with rabbit IgG and half serve as control microwells (i.e., lack antigen). The microwells are incubated with Calibrator, Controls, and Patient Specimens. During the incubation, the RF-IgM present in the test sample binds to the immobilized rabbit IgG. After incubation, unbound antibody is removed by aspiration and washing. To measure bound RF-IgM, rabbit anti-human IgM ($Fc_{5\mu}$ specific) labeled with horseradish peroxidase (HRP) is added to the wells; the plate is then incubataed with the Conjugate and subsequently washed to remove unbound Conjugate. A specific substrate is added and the presence of RF-IgM is detected by a color change that is measured with an ELISA reader. The net absorbance value for the sample is calculated by subtracting the absorbance value for the Control microwell (i.e., a Specimen Blank) from the absorbance value for the antigen-coated microwell. A Conversion Factor (**CF**) then is used to calculate RF-IgM activity (I.U./mL) from the net absorbance value of the sample.

REAGENTS

EL-RF-IgM™ Kit (Cat. No. 301-305), sufficient for 240 EL-RF-IgM tests.

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

Kit Components

1. EL-RF Microwell Plate (Fig. 1)

Half the microwells are coated with rabbit IgG and the other half are saturated with blocking protein (i.e., the wells for Specimen Blanks). A sufficient number of antigen-coated microwells are available to perform 240 EL-RF-IgM tests.

2. Wash Buffer (10X): 10X concentrated buffer with Tween 20. The 1X solution is also used as a Specimen Diluent.

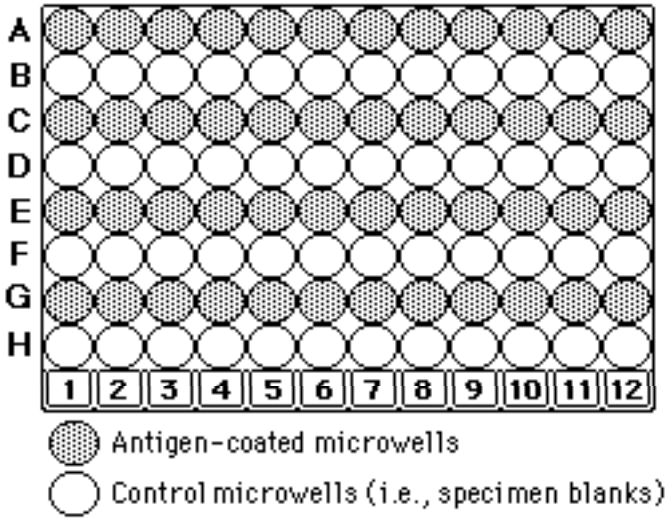

Fig 1. **Distribution of antigen-coated and control microwells for EL-RF 96 microwell plate**

3. Anti-IgM Enzyme Conjugate-Rb: Rabbit anti-human IgM ($Fc_{5\mu}$ specific) coupled with HRP (blue solution).

4. Chromogen: a ready to use solution, containing both the peroxide substrate of Horseradish Peroxidase (HRP) and tetramethylbenzidine as chromogenic indicator.

5. Stop Reagent: 2M phosphoric acid.

6. RF-IgM Calibrator:* Human serum containing RF-IgM in buffer with preservative.

7 RF-IgM Positive Control:* Human serum containing RF-IgM in buffer with preservative.

8. Negative Control:* Human serum with preservative.

9. Sponge: A sponge to absorb any spills.

*Reagents containing sodium azide.

WARNINGS AND PRECAUTIONS

FOR IN VITRO DIAGNOSTIC USE ONLY

IVD

Reagents Containing Human Source Material

CAUTION: Controls and Calibrator 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 and with 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://bmbl.od.nih.gov/

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.

Hazardous Substance Risk and Safety Phrases

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 (Categorized as an **Irritant)**

This product contains 3,3', 5,5'-tetramethyl-benzidine (≤0.05%), a peroxidase cosubstrate which has shown neither mutagenic effects nor carcinogenic effects in laboratory experiments.[8]

Hazardous Substance Risk and Safety Phrases

R36/37/38 – Irritating to eyes, respiratory system, and skin.

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

S36 – Wear suitable protective clothing.

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.

General Precautions and Information

1. Do not use components after expiration date.
2. Wash Buffer (10X), Chromogen, and Stop Reagent are interchangeable among the EL-RF-IgM™ kits. ***All other reagents are kit and lot specific and therefore not interchangeable.***
3. Use gloves while handling specimens and kit reagents; wash hands thoroughly after completion of tests.
4. Do not pipette by mouth.
5. When using the kit, work in a well-ventilated area.
6. Do not eat, drink, or smoke in work areas.
7. Avoid microbial contamination of reagents. If solutions become turbid, they should not be used.
8. Avoid exposure of reagents to excessive heat or light during storage.
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. Check for any crystals in the Wash Buffer (10X) prior to use; redissolve at ~37°C if necessary.

STORAGE AND HANDLING

1. Store all reagents at 2° - 8°C when received. Do not freeze reagents.
2. All reagents are warmed to room temperature (18° - 25°C) for 30 min prior to use.
3. Avoid subjecting reagents to direct sunlight or heat.
4. All reagents, except the 1X Wash Buffer, must be prepared immediately prior to use and discarded afterward. The diluted Wash Buffer (1X), when stored at 2° - 8°C, is stable for 8 weeks.

SPECIMEN COLLECTION

A whole blood specimen should be obtained (in a red top tube) using accepted medical techniques to avoid hemolysis. The blood should be allowed to clot and the serum separated by centrifugation. Unseparated blood can be stored at 18º - 25°C for 24 hours before the separation of serum. The test serum should be clear and non-hemolyzed. Serum samples may be stored at 2º - 8°C for up to 14 days prior to testing. If testing cannot be completed within 14 days of collection, the separated serum must be stored frozen at –20°C. Allow serum to equilibrate to room temperature (18º - 25°C) prior to testing. Do not use serum that has been thawed more than once or that has been heat inactivated. 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

Before starting the assay, read the product insert carefully. Instructions should be followed exactly as they appear in this kit insert to ensure valid results.

A. Materials Provided
(sufficient for 240 RF-IgM tests)

	Item	Quantity
1)	EL-RF 96 Microwell Plates	5
2)	Wash Buffer (10X)	2 x 100 mL
3)	Anti-IgM Enzyme Conjugate-Rb	2 x 30 mL
4)	Chromogen	2 x 27 mL
5)	Stop Reagent	2 x 27 mL
6)	RF-IgM Calibrator	0.35 mL
7)	RF-IgM Positive Control	0.35 mL
8)	Negative Control	0.35 mL

B. Materials Required but not Provided

- 96-place rack with 1.2-mL minitubes (cluster tubes)
- Strips of caps to fit 1.2-mL minitubes
- Precision micropipettors that deliver 5 µL, 100 µL, and 1000 µL (±5%).
- Multichannel (8/12) pipettor that delivers 100 µL and 200 µL
- Disposable plastic pipette tips
- Deionized water (resistivity >1 MOhm) or purified water for irrigation, USP
- Clean wash bottle
- Interval Timer (0 – 60 minutes)
- Multichannel pipette reagent reservoirs
- Single or dual wavelength ELISA reader (with 450 nm filter) for 96-well microtiter plates
- Optional: Multichannel repeat pipettor (50-200 µL)
- Absorbent paper towels

C. Reagent Preparation for Assay

1. **Wash Buffer**: The 10X Wash Buffer (containing Tween 20) 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. ***Note: Check for crystals in the 10X Wash Buffer before its dilution; dissolve any crystals prior to making the dilution. The 1X Wash Buffer is used 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 multichannel pipette reagent reservoir (an excess of 10% of the necessary amount should be measured) no sooner than 5 minutes prior to use. Discard any dispensed but unused Chromogen after completion of the test procedure. Do not pour excess Chromogen back into the stock bottle.
3. **Calibrator, Positive Control, and Negative Control:** All samples (Calibrator, Positive Control, Negative Control, and Patient Specimens,) must be diluted 1:200 prior to use. An example is shown in **Fig. 2c**. Set up the minitube rack as shown and place minitubes in columns in alternate rows. Begin with Calibrator (Cal), Positive Control (PC) and Negative Control (NC) followed by the Patient Specimens. Write on each tube with a waterproof marker: IgM Cal, IgM PC, NC, S1, S2, S3, ...Sn. Pipette 995 µL (or 1 mL) of 1X Wash Buffer into all the marked minitubes, then pipette 5 µL of the appropriate sample into its designated minitube. Cover all minitubes with caps. Place the inverted lid of the tube rack on top of the capped tubes, apply pressure with both hands, and turn upside down ~10 times to uniformly mix the samples. Observe movement of the air bubble to ensure proper mixing.

D. Assay Procedure

1. Bring all reagents to room temperature (18° - 25°C) for 30 minutes prior to use.
2. Configure the strips of microwells as shown in **Fig. 2a** and number the strips on the *rough* tab. To ensure that the strips do not detach from the frame, you may attach some masking tape firmly to the bottom of the plate. Check by inverting and tapping the plate to ensure that the strips are seated securely. The masking tape is removed before adding substrate or before reading OD.

3. Place four 200 µL pipette tips alternately on a multichannel pipettor (minimum 8 channels, see **Fig. 2b**). This arrangement will facilitate simultaneous transfer of samples from the minitubes to each column of microwells. Load the pipettor (with 100 µL of sample from the minitubes, each of 4 channels) and transfer these volumes into the microwells of rows A, C, E, and G (100 µL/well). Using the same tips, pipette 100 µL/well into the microwells of rows B, D, F and H (see **Fig. 2a**). Eject the tips and repeat the same operation for each column of microwells. All samples should be pipetted within 5 minutes.
4. Incubate the plate for 30 - 35 minutes at room temperature (18° - 25°C).
5. For manual washing,, aspirate the microwells and wash the plate 3 times by:
   a. Pipetting 200-400 µL of 1X Wash Buffer into each well.
   b. Aspirating the fluid from each microwell or "flicking" the plate over a sink. After the final wash, invert and vigorously blot plate on absorbent paper.

   (For automatic washing of the plate, follow the instructions provided by the manufacturer, but follow the final wash with manual blotting of the plate on absorbent paper.)
6. With use of a multichannel pipettor, add 100 µL of anti-IgM Enzyme Conjugate-Rb (blue) into all microwells and incubate for 30 - 35 minutes at room temperature (18° - 25°C).
7. Remove Enzyme Conjugate from all wells and wash plate 3 times as described in Step 5. After the final wash, blot plate thoroughly.
8. If masking tape is used, remove it.
9. Pipette 100 µL of Chromogen into all microwells.
10. Incubate the plate for 15 (±1) minutes at room temperature (18° - 25°C). Microwells with Calibrator, Positive Control, and positive Patient Specimens will turn blue.
11. Pipette 100 µL of Stop Reagent into all microwells and mix by gently tapping the plate.
12. Microwells containing the Calibrator, Positive Control, and positive Patient Specimens change from blue to yellow.
13. Firmly seat the microtiter plate in the reader and read the absorbance of each microwell at 450
14. nm. Absorbance values should be measured within 30 minutes of completing the assay. If a dual wavelength ELISA reader is used, set the test wavelength at 450 nm and the reference at 620-690 nm. Follow the 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.
- To ensure that the microwells do not detach from the frame during washing, you may affix some masking tape firmly to the bottom of the plate. Remove this tape prior to reading the plate. To avoid false positive readings, ensure that the bottom of the microwell plate is clean.
- **Crystallization of Reagents:** The 10X Wash Buffer may crystallize at 2° - 8°C. To dissolve the crystals, warm the bottle at 37°C until crystals have dissolved. ***Failure to dissolve crystals before use of the 10X Wash Buffer will lead to erroneous results***.
- **Washing**: Each row of wells may be washed manually using a multichannel pipettor or a multichannel repeat pipettor. To remove the fluid from the plates, the wells should be aspirated or the plates should be "flicked" over a sink. Alternatively, a commercial semi-automated washing system may be used following the instructions provided by the manufacturer. When using either washing technique, blotting the wells on absorbent paper after the final wash is required.

NOTE: Insufficient washing produces inaccurate test results.

- **Pipetting**: To avoid cross-contamination and sample carryover, Calibrator, Positive Control, Negative Control, and Patient Specimens MUST be pipetted using separate pipette tips. A multichannel pipettor may be used to pipette Enzyme Conjugate, Wash Buffer, Chromogen, and Stop Reagent.

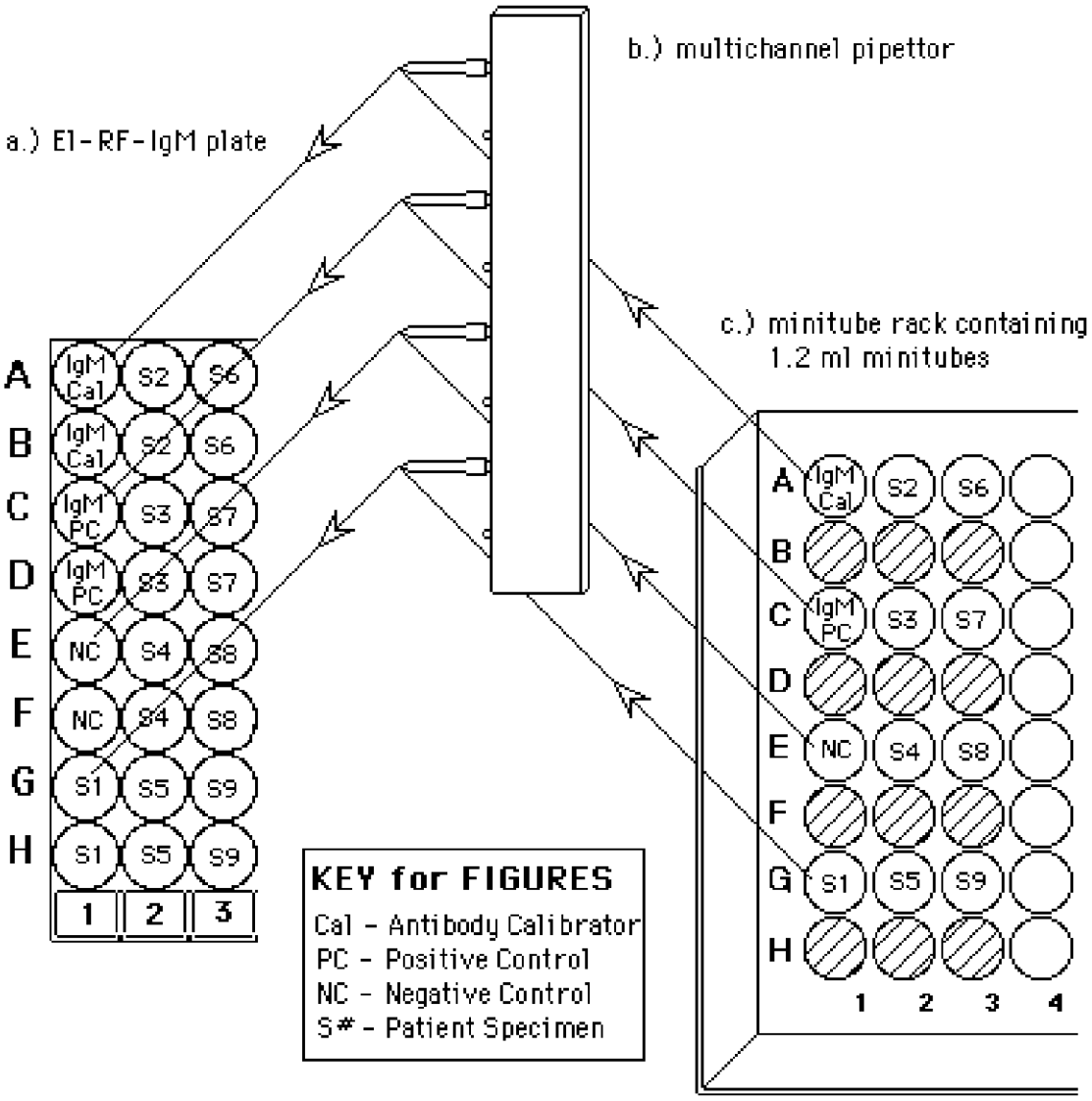

Fig 2. Arrangement of minitubes and EL-RF 96 microwell plate for EL-RF-IgM Assay. **(a)** Section of EL-RF 96 microwell plate; **(b)** Multichannel pipettor for transferring 100 µL aliquots; **(c)** Minitube rack containing 1.2-mL minitubes filled with diluted Calibrator, Positive Control, Negative Control, and Specimens.

QUALITY CONTROL

1. **Specimen Blank:** For a Specimen Blank, the absorbance value should be less than 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 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 0.3.
2. Elevated values for Specimen Blanks may be caused by hypergammaglobulinemia. If the Specimen Blank value is still high after repetition of the test with the extra dilution of the Specimen, contact the manufacturer.
3. **Calibrator:** The Calibrator should have a net absorbance value that is within the range shown on the Data Sheet. The TheraTest RF-IgM Unit (I.U.) is traceable to the World Health Organization Reference Preparation for Rheumatoid Factor.

4. **Positive and Negative Controls:** Positive and Negative Controls should be run with each test procedure. The acceptable ranges of Unit values (I.U./mL) for the Positive and Negative Controls are listed in the Data Sheet. When the results for Positive and Negative Controls are not within the specified ranges, the run must be repeated.

   Note: *When a plausible explanation for an aberrant result is not apparent, the test must be repeated. If corrective action and repeated testing fail to solve the problem, contact the manufacturer.*

5. **Patient Specimens:** If the net absorbance value for a Patient Specimen exceeds 2.0 OD, retest by further diluting the Specimen 1:10 (final, 1:2000) and repeat the test procedure. To obtain I.U./mL, use the following formula:

   CF x [Net Abs.(of 10-fold diluted Specimen)] x [10] = I.U./mL in Specimen

6. **Testing of QC Samples:** It is recommended that the CDC/WHO RF-IgM Standard be tested with each lot of EL-RF-IgM™ kits. The WHO standard contains 1,000 I.U./mL of RF-IgM activity. To obtain an acceptable absorbance, dilute the Standard 1:2000 in Specimen Diluent (1X Wash Buffer) before testing it. Results of 1,000 I.U./mL ± 25% are acceptable.

RESULTS

A. Calculation of Results

Calculating RF-IgM Net Absorbance

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 control microwell (i.e., Specimen Blank) from the absorbance value of its antigen-coated microwell in each pair.

NOTE: Microwells in rows A, C, E and G contain antigen while microwells in rows B, D, F and H are their respective controls (i.e., the Specimen Blanks).

Example:

Absorbance for Sample in antigen-coated microwell = 1.250
Absorbance for Sample in control microwell = 0.250
Net Absorbance = 1.250 – 0.250 = 1.000

Calculating RF-IgM Units from a One-Point Calibrator

Determine the net absorbance values for all wells of the RF-IgM test plate (Calibrator, Controls, and Patient Specimens). The number of I.U./mL of RF-IgM in the Calibrator is given on the Data Sheet. A Conversion Factor (**CF**) can then be determined as follows:

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

Then,

CF x Net $Abs_{(Sample)}$ = I.U./$mL_{(Sample)}$ of RF-IgM

Example:

Calibrator = 180 I.U./mL
Net Absorbance of Calibrator = 1.2
Net Absorbance of Sample #1= 0.6

CF = 180 ÷ 1.2 = 150

CF x Net Abs Sample = I.U./mL of Sample
150 x 0.6 = 90 I.U./$mL_{(Sample\ \#1)}$ of RF-IgM

The Conversion Factor (**CF**) **must** be calculated with **each** test procedure. If the net absorbance value for a Patient Specimen exceeds 2.0, retest by further diluting the Specimen 1:10 (final, 1:2000) and repeat the test procedure. To obtain Unit activity for the 1:2000 dilution, use the following formula:

CF x (Net Abs. of Sample x 10) = I.U./mL RF-IgM

B. Limitations of the Procedure

The EL-RF-IgM™ test should not be performed on grossly hemolysed, microbially contaminated, or grossly lipemic samples. Only sera have been tested. 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 exam and other data) is essential to diagnose RA. Furthermore, a negative result does not exclude RA.

EXPECTED VALUES

Acceptable net absorbance and the given Unit value for the Calibrator as well as acceptable Unit ranges for the Positive and Negative Controls are shown on the Data Sheet enclosed with the kit.

Normal limits have been defined based on results obtained from 131 blood bank donors; the upper limit of normal for RF-IgM was defined as 25 I.U./mL (95 percentile). Expected values for positive Patient Specimens are included in the Data Sheet. For example, the values for 34 RA patients ranged from 5 to 5700 I.U./mL.

GUIDE TO INTERPRETATION

Elevated serum levels of RF-IgM are present frequently in patients with RA. Nevertheless, abnormal values of RF-IgM are also prevalent in the aged population (10-15%) and have been detected in a number of other disorders with prevalence values as follows: 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%).

PERFORMANCE DATA

A. Accuracy: RF-IgM values from EL-RF-IgM™ tests were compared to values from a commercially available nephelometric test. Thirty-four RA and 31 normals were tested. Table 1A shows the number of individual positive and negative tests by each method whereas Table 1B shows the calculated sensitivities and specificities. The RF-IgM values obtained by the two methods for RA samples were plotted and linear regression analysis yielded:

$y = 2.6x - 175.3$ where $r = 0.90$.

Table 1A. Comparison of positive and negative results for sera tested simultaneously by EL-RF-IgM tests and by a nephelometric method.

		EL-RF-IgM kit	
		Positive	Negative
Nephelometry	Positive	30	0
	Negative	1	34

Table 1B. Sensitivity and specificity of the EL-RF-IgM test versus a nephelometric method

	EL-RF-IgM Kit	Nephelometry
Sensitivity	30/(30+4)=88%	29/(29+5)=85%
Specificity	30/(30+1)=96%	30/(30+1)=96%

B. Precision: Within-run precision was performed with Specimens that were known to contain one of three different levels of RF-IgM reactivity. 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 in 12 separate runs (plates); the coefficient of variation was less than 10%.

C. Test Specificity: Test specificity of the TheraTest EL-RF-IgM test system was determined by inhibiting autoantibody binding with soluble rabbit or human IgG. The Calibrator was incubated with IgG and the mixture was subsequently incubated with antigen-coated wells. Over 80% of the absorbance was inhibited, indicating that the autoantibody identified was indeed directed against IgG.

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TROUBLESHOOTING

Problem	Possible Causes	Solution(s)
OD values of Specimen Blanks are >0.3.	1. Chromogen may be contaminated.	1. Read OD of Chromogen + Stop reagent (100 µL each in a microwell); if OD >0.1, discard Chromogen, use new bottle & repeat test.
	2. Improper storage or aging of Specimens.	2. Check storage conditions for reagents & Specimens.
Calibrator OD value is out of range.	1. Incorrect incubation temperature or timing.	1. Check that temperature was correct. Check that time was correct. See "Poor Precision" (below) No. 2-4. Repeat test.
	2. Calibrator not properly mixed.	2. Mix all reagents before use.
	3. Improper dilution.	3. Repeat test with attention to dilutions.
	4. Wavelength of filter incorrect.	4. Change filter to 450 ± 5 nm.
	5. Contamination of Calibrator in well.	5. Repeat test; pipette carefully.
	6. Calibrator Blank OD >0.3.	6.a) an excess volume of Conjugate was added to the Calibrator Blank well. b) the incubation time was too long. c) insufficient washing. d) a damaged or dirty well. e) Chromogen is contaminated, replace.
OD value for Calibrator &/or Unit Value for Positive Control (PC) show sudden downward shifts.	A sudden downward shift suggests: 1. improper filter selection for ELISA reader. 2. a reduced volume or improper dilution of sample or one of the reagents. 3. a lower than usual incubation temperature. 4. a shortened incubation time. 5. poor quality water for 1X Wash Buffer preparation.	1. Change filter to 450 nm for ELISA reader; reread plate if time elapsed since development is <30 min. 2. Review sample & reagent volumes used for the test; make corrections, if needed, and rerun test. 3. Check that room temperature (RT) was between 18º & 25ºC, and that reagents were warmed to RT. 4. Assure that the timer functions properly and that timed interval settings are correct. 5. Use bottled purified water for irrigation, USP. **Note:** For errors other than wrong filter, repeat the test.
OD values for Calibrator and Unit values for PC show downward trends.	A downward trend on either or both sets of values suggests possible reagent decay (antigen-coated wells, Calibrator, PC, Conjugate, and/or Substrate).	Assure that microwells and reagents are properly stored at 2º - 8ºC (i.e. improperly closed pouches, loose caps, etc.). Check empty microwells for condensate and if present, contact manufacturer. Consider decay of Calibrator or PC if only one reagent shows downward trend. If OD and Unit values are within acceptable ranges for Calibrator and PC, accept test results. Use new reagent(s) and repeat the test if a reagent error is suspected.

TROUBLESHOOTING (CONT'D)

Problem	Possible Causes	Solution(s)
OD values for Calibrator and/or Unit values for PC show sudden upward shifts.	A sudden upward shift suggests: 1. an increased volume of the sample or one of the reagents. 2. a greater than usual incubation temperature. 3. a lengthened incubation time. 4. improper mixing of reagents. 5. contamination of the well with Conjugate.	1. Review sample and reagent volumes used for the test; make corrections, if needed, and rerun test. 2. Check that Room Temperature (RT) is in the 18º to 25º range; adjust if necessary. 3. Assure that the timer functions properly and that timed interval settings are correct. 4. Mix all reagents before use. 5. Repeat the test; pipette carefully.
Positive and/or Negative Control Unit values are out of range.	1. Incorrect temperature or timing for incubation. 2. Incorrect volumes of reagents pipetted. 3. Reagents were not properly mixed. 4. Cross-contamination of Controls. 5. Optical pathway not clean.	1. Check incubation temperature and timing. If error occurred, correct problem & rerun test. 2. Assess accuracy/precision of pipettes and assure correct volumes were delivered. If error, rerun test. 3. Mix all reagents before use. 4. Pipette carefully, changing tips for each reagent. 5. Check for moisture, dirt, or air bubbles in or on wells. Tap plate, wipe bottom and reread.
No color development.	1. One or more reagents not added, or added in wrong sequence. 2. Antigen coated wells are inactive. 3. Improper dilution of Wash Buffer concentrate.	1. Recheck procedure. Be sure Stop Reagent is added last. Check for unused reagent(s). 2. Check for obvious moisture in unused wells – an indicator of condensate from opening a cold pouch. Use microwells from a new pouch and repeat test. 3. Prepare new 1X Wash Buffer and repeat test. Do not confuse Stop Reagent with Wash Buffer Concentrate (similar bottles) when preparing 1X Wash Buffer. Use of diluted Stop Reagent in place of 1X Wash Buffer will destroy color development. **Note:** Repeat tests on Calibrator and Controls first for a check on good performance after changes. Contact manufacturer if trouble persists.
All test results are yellow.	1. One or more reagents may be contaminated; focus first on Chromogen. 2. Contaminated buffers and reagents. 3. Wash Buffer (1X) contaminated or improper washing due to crystal formation in the 10X Wash Buffer conc. 4. Improper dilution of serum/Controls.	1. Check absorbance of 100µL unused Chromogen + 100µL Stop Reagent; discard if absorbance >0.1. 2. Check all solutions for turbidity. Discard turbid reagents and repeat test with new reagents. 3. Use clean container. Check quality of water used to prepare buffer; check for crystal formation in the Wash Buffer concentrate (***dissolve crystals before preparing 1X Wash Buffer***). 4. Check by measuring residual volume of diluted Specimen. Remember that dilutions of Controls and Specimens are 5 µL + 195 µL. Make corrections and repeat test.

TROUBLESHOOTING (CONT'D)

Problem	Possible Causes	Solution(s)
Poor precision	1. Pipettor delivery CV greater than 5% or samples not added slowly.	1. Check calibration of pipettor. Use reproducible technique.
	2. Serum or reagents not mixed sufficiently; reagents not at RT prior to addition.	2. Mix all reagents gently but thoroughly and equilibrate to RT.
	3. Reagent addition taking too long; inconsistency in timing intervals; air bubbles in pipette tips.	3. Develop consistent uniform technique and avoid splashing; use multi-tip device or autodispenser to decrease reagent delivery time.
	4. Air currents blowing over plate during incubations.	4. Cover plate or place plate in chamber.
	5. Optical pathway not clean.	5. Wipe bottom of plate with soft tissue. Check instrument light source and detector for dirt.
	6. Instrument not equilibrated before readings were taken.	6. Check instrument warm-up procedure.
	7. Washing not consistent; trapped bubbles; liquid left in wells at end of wash cycle.	7. Use only acceptable washing devices. Lengthen timing delay on automated washing devices. Check that all wells are filled and aspirated uniformly. Dispense 1X Wash Buffer above level of reagents previously added to wells.
	8. Improper pipetting.	8. Avoid air bubbles in pipette tips. Develop consistent pipetting technique.

Abbreviated Test Procedure EL-RF-IgM

1. **Dilute Calibrator, Positive Control, Negative Control, and Specimens as required for IgM assays(1:200).**
   a) **Add 995 µL (1 mL) of 1X Wash Buffer (Specimen Diluent) to minitubes in rows A, C, E, and G (No. of minitubes filled = 3 + No. of Patient Specimens).**
   b) **Add 5 µL each of Calibrator and Positive Control to the appropriate dilution minitubes: Cal (A1), PC (C1).**
   c) **Add 5 µL of the Negative Control to the dilution minitube (E1).**
   d) **Add 5 µL of each Patient Specimen to appropriate minitubes: S1 into (G1), S2 into (A2), S3 into (C2), S4 into (E2), S5 into (G2), etc.**
   e) **Mix all dilution minitube contents well.**
2. **Transfer 100 µL of diluted Calibrator, Positive Control, Negative Control and Specimen #1 from the minitubes in Tube Rack Column 1 into the designated paired wells (antigen-coated and Blank wells) of the Plate, Column 1 (Refer to Fig 2.).**
3. **Transfer 100 µL of Specimens #2, #3, #4, and #5 from the minitubes in Tube Rack Column 2 into the designated paired wells (antigen-coated and Blank wells) of the Plate, Column 2 (Refer to Fig 2.). Continue transfers until all diluted Specimens are transferred.**
4. **Incubate the plate for 30±5 minutes at room temperature (18º - 25ºC).**
5. **Wash the wells three times with 1X Wash Buffer.**
6. **Add 100 µL of the Enzyme Conjugate to each of the sample wells (Calibrator, PC, NC, and Patient Specimen wells).**
7. **Incubate the plate for 30 (±5) minutes at room temperature (18º - 25ºC).**
8. **Wash the wells three times with 1X Wash Buffer.**
9. **Add 100 µL Chromogen to each well.**
10. **Incubate plate for 15 (±1) minutes at room temperature (18º - 25ºC).**
11. **Add 100 µL Stop Reagent to each well.**
12. **Read the absorbance at 450 nm, reference 620-690 nm, within 30 minutes.**

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