1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic relapsing inflammatory arthritis usually affecting multiple joints with a varying degree of systemic involvement. RA is a highly variable disease that can range from a mild illness of brief duration to a progressive destructive polyarthritis associated with systemic vasculitis. It is estimated that RA affects 0.5 % to 1 % of the population worldwide and is two to three times more common in females than in males. The prevalence of RA increases with age, peaking at 35-45 years of age. The etiology of RA is not fully understood. Evidence points to a complex interplay between environmental and genetic factors. The main genetic risk factor of RA is a certain form of the HLA-DR (human leukocyte antigen, subtype DR) allele. Untreated, RA leads to bone erosion, cartilage damage, joint destruction, functional limitation and severe disability, and has a significant impact on health-related quality of life. Joint destruction in RA begins within a few weeks of symptom onset; early treatment decreases the rate of disease progression. Therefore, early diagnosis and suitable therapy are of decisive importance for the prognosis of RA. Therapeutic goals include preservation of function and quality of life, minimization of pain and inflammation, joint protection, and control of systemic complications. A characteristic of RA is the presence of certain autoantibodies collectively known as rheumatoid factors (RF). Rheumatoid factors are a subset of antiglobulins with antibody activity directed against antigenic sites in the Fc region of immunoglobin G. They exist as IgA-, IgG-and IgM-isotypes, with IgM and IgG being the most common. Rheumatoid factors have been reported to occur in around 70-80 % of patients diagnosed with RA. They may occur early in the disease and can even precede the development of clinical manifestations by several years. The concentration of RF tends to be highest when the disease peaks and tends to decrease during prolonged remission. However, these factors are not unique to RA. Positive RF test results may also be seen in healthy people and in people with viral infections and a number of other diseases such as: infectious mononucleosis, endocarditis, tuberculosis, syphilis, liver disease, sarcoidosis and systemic lupus erythematosus. Therefore, the diagnosis cannot be made by laboratory tests alone. Clinical exams, X-rays and abnormal laboratory values (RF, erythrocyte sedimentation rate, C-reactive protein, anti-CCP) are used to determine a diagnosis of rheumatoid arthritis and assess treatment effectiveness. The presence of rheumatoid factors may be detected by latex agglutination, nephelometry and several quantitative immunoassays, e.g. radioimmunoassays and ELISA (Enzyme-linked Immunosorbent Assay).

2. INTENDED USE

The GenWay Rheumatoid Factor IgM ELISA is intended for the quantitative determination of IgM rheumatoid factors in human serum or plasma (citrate).

3. PRINCIPLE OF THE ASSAY

The quantitative immunoenzymatic determination of IgM rheumatoid factors is based on the ELISA (Enzyme-linked Immunosorbent Assay) technique. Microtiter strips are precoated with Fc fragments of human immunoglobulin G to bind corresponding antibodies of the specimen. After washing the wells to remove all unbound sample material horseradish peroxidase (HRP) labelled anti-human IgM conjugate is added. This conjugate binds to the captured rheumatoid factors. The immune complex formed by the bound conjugate is visualized by adding Tetramethylbenzidine (TMB) substrate which gives a blue reaction product. The intensity of this product is proportional to the amount of IgM rheumatoid factors in the specimen. Sulphuric acid is added to stop the reaction. This produces a yellow endpoint colour. Absorbance at 450 nm is read using an ELISA microwell plate reader.

4. MATERIALS

4.1. Reagents supplied

Fc Fragment Coated Wells: 12 breakapart 8-well snap-off strips coated with Fc fragments of human immunoglobulin G; in resealable aluminium foil.

Sample Diluent**: 1 bottle containing 100 ml of buffer for sample dilution; pH 7.2 ± 0.2, coloured yellow; ready to use; white cap.

Stop Solution: 1 bottle containing 15 ml sulphuric acid, 0.2 mol/l; ready to use; red cap.

Washing Solution (20x conc.)*: 1 bottle containing 50 ml of a 20-fold concentrated buffer for washing the wells; pH 7.2 ± 0.2; white cap.

Rheumatoid Factor anti-IgM Conjugate**: 1 bottle containing 20 ml of peroxidase labelled antibodies to human IgM; coloured red; ready to use; black cap.

TMB Substrate Solution: 1 bottle containing 15 ml 3,3’,5,5’-tetramethylbenzidine (TMB); ready to use; yellow cap.

Rheumatoid Factor IgM Negative Control***: 1 bottle containing 2 ml control solution; coloured yellow; ready to use; blue cap.

Rheumatoid Factor IgM Positive Control***: 1 bottle containing 2 ml control solution; coloured yellow; ready to use; red cap.

Rheumatoid Factor IgM Standards**: 6 vials, each containing 2 ml; coloured yellow; ready to use; yellow cap.

Standard A: 0 IU/ml
Standard B: 3 IU/ml
Standard C: 10 IU/ml
Standard D: 30 IU/ml
Standard E: 100 IU/ml
Standard F: 300 IU/ml

* contains 0.1 % Bronidox L after dilution ** contains 0.2 % Bronidox L *** contains 0.1 % Kathon

4.2. Materials supplied

1 Strip holder
1 Cover foil
1 Test protocol
1 Distribution and identification plan
4.3. Materials and Equipment needed
ELISA microwell plate reader, equipped for the measurement of absorbance at 450/620 nm
Incubator 37 °C
Manual or automatic equipment for rinsing wells
Pipettes to deliver volumes between 10 and 1000 µl
Vortex tube mixer
Deionised or (freshly) distilled water
Disposable tubes
Timer

5. STABILITY AND STORAGE
The reagents are stable up to the expiry date stated on the label when stored at 2...8 °C.

6. REAGENT PREPARATION
It is very important to bring all reagents, samples and standards to room temperature (20…25 °C) before starting the test run!

6.1. Coated Snap-off Strips
The ready to use breakapart snap-off strips are coated with Fc fragments of human immunoglobulin G. Store at 2…8 °C. Immediately after removal of strips, the remaining strips should be resealed in the aluminium foil along with the desiccant supplied and stored at 2…8 °C; stability until the expiry date.

6.2. Rheumatoid Factor anti-IgM Conjugate
The bottle contains 20 ml of a solution with anti-human IgM horseradish peroxidase, buffer, stabilizers, preservatives and an inert red dye. The solution is ready to use. Store at 2…8 °C. After first opening stability until the expiry date when stored at 2…8 °C.

6.3. Rheumatoid Factor IgM Controls
The bottles labelled with Negative and Positive Control contain a ready to use control solution.
Negative Control: < 5 IU/ml Positive Control: > 20 IU/ml
It contains 0.1 % Kathon and has to be stored at 2...8 °C. After first opening stability until the expiry date when stored at 2…8 °C.

6.4. Rheumatoid Factor IgM Standards
The vials labelled with Standard A, B, C, D, E and F contain a ready to use standard solution calibrated in accordance with the 1st British Standard 64/002. The standards have the following concentrations in international units (IU/ml):
Standard A: 0 IU/mlStandard B: 3 IU/mlStandard C: 10 IU/mlStandard D: 30 IU/mlStandard E: 100 IU/mlStandard F: 300 IU/ml
The solutions have to be stored at 2…8 °C and conta in 0.1 % Kathon. After first opening stability until the expiry date when stored at 2…8 °C.

6.5. Sample Diluent
The bottle contains 100 ml phosphate buffer, stabilizers, preservatives and an inert yellow dye. It is used for the dilution of the patient specimen. This ready to use solution has to be stored at 2…8 °C. After first opening stability until the expiry date when stored at 2…8 °C.

6.6. Washing Solution (20x conc.)
The bottle contains 50 ml of a concentrated buffer, detergents and preservatives. Dilute washing solution 1 + 19; e. g. 10 ml Washing Solution + 190 ml fresh and germ free redistilled water. The diluted buffer will keep for 5 days if stored at room temperature. Crystals in the solution disappear by warming up to 37 °C in a water bath. After first opening stability until the expiry date when stored at 2…8 °C.

6.7. TMB Substrate Solution
The bottle contains 15 ml of a tetramethylbenzidine/hydrogen peroxide system. The reagent is ready to use and has to be stored at 2…8 °C, away from the light. The solution should be colourless or could have a slight blue tinge. If the substrate turns into blue, it may have become contaminated and should be thrown away. After first opening stability until the expiry date when stored at 2…8 °C.

6.8. Stop Solution
The bottle contains 15 ml 0.2 M sulphuric acid solution (R 36/38, S 26). This ready to use solution has to be stored at 2…8 °C. After first opening stability until the expiry date.

7. SPECIMEN COLLECTION AND PREPARATION
Use human serum or plasma (citrate) samples with this assay. If the assay is performed within 5 days after sample collection, the specimens should be kept at 2…8 °C; otherwise they should be aliquoted and stored deep-frozen (-20… -70 °C). If samples are stored frozen, mix thawed samples well before testing. Avoid repeated freezing and thawing. Heat inactivation of samples is not recommended.

7.1. Sample Dilution
Before assaying, all samples should be diluted 1 + 100 with Sample Diluent. Dispense 10 µl sample and 1 ml Sample Diluent into tubes to obtain a 1 + 100 dilution and thoroughly mix with a Vortex.

8. ASSAY PROCEDURE

8.1. Test Preparation
Please read the test protocol carefully before performing the assay. Result reliability depends on strict adherence to the test protocol as described. If performing the test on ELISA automatic systems we recommend increasing the washing steps from three to five and the volume of Washing Solution from 300 µl to 350 µl to avoid washing effects. Prior to commencing the assay, the distribution and identification plan for all specimens and standards should be carefully established on the result sheet supplied in the kit. Select the required number of microtiter strips or wells and insert them into the holder.
Please allocate at least:
1 well (e.g. A1) for the substrate blank, 6 wells (e.g. B1, C1, etc.) for Standard A, B, C, D, E and F, 1 well (e.g. H1) for the Negative Control and 1 well (e.g. A2) for the Positive Control

It is recommended to determine standards, controls and patient samples in duplicate.

Perform all assay steps in the order given and without any appreciable delays between the steps.

A clean, disposable tip should be used for dispensing each standard, control and sample.

Adjust the incubator to 37 ± 1 °C.

1. Dispense 100 µl of each Standard (A, B, C, D, E and F), controls and diluted samples into their respective wells. Leave well A1 for the substrate blank.
2. Cover wells with the foil supplied in the kit.
3. **Incubate for 30 min at 37 ± 1 °C.**
4. When incubation has been completed, remove the foil, aspirate the content of the wells and wash each well three times with 300 µl of Washing Solution. Avoid overflows from the reaction wells. The soak time between each wash cycle should be > 5 sec. At the end carefully remove remaining fluid by tapping strips on tissue paper prior to the next step!

   *Note: Washing is critical! Insufficient washing results in poor precision and falsely elevated absorbance values.*

1. Dispense 100 µl Rheumatoid Factor anti-IgM Conjugate into all wells except for the blank well (e.g. A1). Cover with foil.
2. **Incubate for 30 min at room temperature (20…25 °C). Do not expose to direct sunlight.**
3. Repeat step 4.
4. Dispense 100 µl TMB Substrate Solution into all wells.
5. **Incubate for exactly 15 min at room temperature (20…25 °C) in the dark.**
6. Dispense 100 µl Stop Solution into all wells in the same order and at the same rate as for the TMB Substrate Solution.

Any blue colour developed during the incubation turns into yellow.

11. Measure the absorbance of the specimen at 450/620 nm within 30 min after addition of the Stop Solution.

8.2. Measurement Adjust the ELISA Microwell Plate Reader to zero using the substrate blank in well A1.

If - due to technical reasons - the ELISA reader cannot be adjusted to zero using the substrate blank in well A1, subtract the absorbance value of well A1 from all other absorbance values measured in order to obtain reliable results!

**Measure the absorbance** of all wells at 450 nm and record the absorbance values for each control and patient sample in the distribution and identification plan.

Where applicable calculate the **mean absorbance values** of all duplicates.

9. RESULTS

9.1. Assay Validation Criteria

In order for an assay to be considered valid, the following criteria must be met:

- **Substrate blank** in A1: Absorbance < 0.100
- **Standard A** in B1: Absorbance < 0.200
- **Standard B** in C1: Absorbance > **Standard A**
- **Standard C** in D1: Absorbance > **Standard B**
- **Standard D** in E1: Absorbance > 0.100
- **Standard E** in F1: Absorbance > 0.400
- **Standard F** in G1: Absorbance > 1.000
- **Negative Control** in H1: Result < 5 IU/ml
- **Positive Control** in A2: Result > 20 IU/ml


If these criteria are not met, the test is not valid and must be repeated.

9.2. Calculation of Results

In order to obtain **quantitative results in IU/ml** plot the (mean) absorbance values of the Standards A – F (y-axis, linear) on graph paper in a system of coordinates against their corresponding concentrations (x-axis, logarithmic) and draw a standard calibration curve. Read results from this standard curve employing the (mean) absorbance values of each patient specimen and control.

All suitable computer programs available can be used for automated result reading and calculation.

9.3. Interpretation of Results

Normal value ranges for this ELISA should be established by each laboratory based on its own patient populations in the geographical areas serviced.

The following values should be considered as a guideline:
- **negative**: < 10 IU/ml grey zone: 10 -15 IU/ml **positive**: > 15 IU/ml

Positive results should be verified concerning the entire clinical status of the patient.
10. SPECIFIC PERFORMANCE CHARACTERISTICS

10.1. Precision

<table>
<thead>
<tr>
<th>Intraassay</th>
<th>n</th>
<th>Mean (OD)</th>
<th>CV (%)</th>
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<tbody>
<tr>
<td>Pos Serum</td>
<td>24</td>
<td>1.170</td>
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<tr>
<td>Pos. Serum</td>
<td>24</td>
<td>2.127</td>
<td>2.1</td>
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<tr>
<td>Neg. Serum</td>
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<table>
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<th>Interassay</th>
<th>n</th>
<th>Mean (IU/ml)</th>
<th>CV (%)</th>
</tr>
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<tr>
<td>Pos. Serum</td>
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<td>33.7</td>
<td>5.3</td>
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<tr>
<td>Pos. Serum</td>
<td>12</td>
<td>148.6</td>
<td>6.4</td>
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</tbody>
</table>

10.2. Diagnostic Specificity
The diagnostic specificity is defined as the probability of the assay of scoring negative in the absence of the specific analyte. It is 94.1 %.

10.3. Diagnostic Sensitivity
The diagnostic sensitivity is defined as the probability of the assay of scoring positive in the presence of the specific analyte. It is 98.0 %.

10.4. Analytical Sensitivity
The analytical sensitivity – defined as the apparent concentration of the analyte that can be distinguished from the zero calibrator – is < 0.5 IU/ml.

10.5. Interferences
Interferences with hemolytic, lipemic or icteric sera are not observed up to a concentration of 10 mg/ml hemoglobin, 5 mg/ml triglycerides and 0.5 mg/ml bilirubin.

11. LIMITATIONS OF THE PROCEDURE
Bacterial contamination or repeated freeze-thaw cycles of the specimen may affect the absorbance values.

12. PRECAUTIONS AND WARNINGS

Only for research use.

All components of human origin used for the production of these reagents have been tested for anti-HIV antibodies, anti-HCV antibodies and HBsAg and have been found to be non-reactive. Nevertheless, all materials should still be regarded and handled as potentially infectious.

Do not interchange reagents or strips of different production lots.

No reagents of other manufacturers should be used along with reagents of this test kit.

Do not use reagents after expiry date stated on the label.

Use only clean pipette tips, dispensers, and lab ware.

Do not interchange screw caps of reagent vials to avoid cross-contamination.

Close reagent vials tightly immediately after use to avoid evaporation and microbial contamination.

After first opening and subsequent storage check conjugate and control vials for microbial contamination prior to further use.

To avoid cross-contamination and falsely elevated results pipette patient samples and dispense conjugate without splashing accurately to the bottom of wells.

The GenWay ELISA is only designed for qualified personnel who are familiar with good laboratory practice.

WARNING: In the used concentration Bronidox L has hardly any toxicological risk upon contact with skin and mucous membranes!

WARNING: Sulphuric acid irritates eyes and skin. Keep out of the reach of children. Upon contact with the eyes, rinse thoroughly with water and consult a doctor!

12.1. Disposal Considerations

Residues of chemicals and preparations are generally considered as hazardous waste. The disposal of this kind of waste is regulated through national and regional laws and regulations. Contact your local authorities or waste management companies which will give advice on how to dispose hazardous waste.
Rheumatoid Factor IgM

Assay Preparation

Prepare reagents and samples as described. Establish the distribution and identification plan for all specimens and standards on the result sheets supplied in the kit.

Select the required number of microtiter strips or wells and insert them into the holder.

### Assay Procedure

<table>
<thead>
<tr>
<th></th>
<th>blank</th>
<th>Standard A -F</th>
<th>Negative Control</th>
<th>Positive Control</th>
<th>Sample (diluted 1+100)</th>
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<tr>
<td>blank</td>
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<tr>
<td>Standard A -F</td>
<td>-  100 µl</td>
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<tr>
<td>Negative Control</td>
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<td>100 µl</td>
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<tr>
<td>Positive Control</td>
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<td>100 µl</td>
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<tr>
<td>Sample (diluted 1+100)</td>
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<td>100 µl</td>
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</table>

Cover wells with foil supplied in the kit. **Incubate for 30 min at 37 °C.** Wash each well three times with 300 µl diluted Washing Solution.

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<tr>
<th></th>
<th>Conjugate</th>
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<th>100 µl</th>
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</table>

Cover wells with foil supplied in the kit. **Incubate for 30 min at 20….25 °C.** Wash each well three times with 300 µl diluted Washing Solution.

<table>
<thead>
<tr>
<th></th>
<th>TMB Substrate</th>
<th>100 µl</th>
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**Incubate for exactly 15 min at room temperature in the dark.**

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<tr>
<th></th>
<th>Stop Solution</th>
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Photometric measurement at 450 nm (reference wavelength: 620 nm)

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