Semi-quantitative determination of IgG class antibodies against IFI16 in human serum or plasma

**INTENDED USE**
Eagle Biosciences Anti IFI16 ELISA Assay Kit is an enzyme-linked immunosorbent assay (ELISA) designed for the semi-quantitative measurement of IgG class antibodies directed against the IFI16 antigen in human serum or plasma. Anti IFI16 kit is intended for research use only and is not intended for diagnostic procedures.

**1. CLINICAL SIGNIFICANCE**
Anti IFI16 ELISA Assay Kit is a semi-quantitative, enzyme-linked immunosorbent assay (ELISA) for the detection of IgG class antibodies directed against the IFI16 antigen in human serum or plasma. Systemic autoimmune disease is characterized by the presence of circulating autoantibodies. Systemic Sclerosis (SSc) is an autoimmune multisystem connective tissue disorder characterized by inflammatory and fibrotic processes, affecting the skin, blood vessels and internal organs. The two widely recognised subsets of SSc are limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc). These two forms of SSc present divergent patterns of internal organ involvement, clinical course and autoantibody profiles, as defined by the Le Roy criteria (LeRoy et al. 2001). SSc is characterised by the occurrence of antinuclear autoantibodies. Anti-centromere (ACA), anti-DNA topoisomerase-I (anti-Scl70) are the most common autoantibodies and, along with anti-RNA polymerases (RNAP I-III), are specific to SSc. Several cellular activities have been assigned to IFI16, including roles in antiviral, inflammatory and apoptotic processes. Anti IFI16 antibodies have been described in the sera of patients affected by systemic autoimmune disorders (reviewed in Mondini et al. 2007). Although the subcellular localization of the IFI16 autoantigen is well recognized as nuclear, Anti IFI16 cannot be classified as a classical anti-nuclear antibody (ANA), because it lacks HEp-2 staining, the gold standard for identifying ANAs (Costa et al. 2011). In patients with a diagnosis of Systemic Sclerosis (SSc), Anti IFI16 antibodies have been detected with a prevalence up to 29% (Mondini et al. 2006, Costa et al. 2011). A subgroup of SSc patients (34% of the entire population, Costa et al. 2011) tests negative for ACA and anti-Scl70 (double negative patients, dnSSc). Anti IFI16 antibodies are found in up to 30% of the dnSSc patients and, in this subgroup, are significantly associated with the limited cutaneous SSc subset (Mondini et al. 2006, Costa et al. 2011). Moreover, Anti IFI16 have been shown to segregate with a different population in respect to anti-RNAP III (Costa et al. 2011). Thus, the detection of Anti IFI16 can aid in differentiating the SSc subtypes in conjunction with clinical and laboratory findings, being useful for disease classification in patients negative for other serological markers of SSc (Costa et al. 2011).

**2. PRINCIPLE**
Anti IFI16 ELISA Assay Kit is based on the binding of antibodies present in calibrators, controls or pre-diluted samples to the IFI16 antigen coated on the inner surface of the microplate wells. After 30 minutes incubation the microplate is washed with wash buffer to remove the non-reactive serum components. An anti-human-IgG hors eradish peroxidase conjugate solution recognizes IgG class antibodies bound to the immobilized antigens. After a 30 minutes incubation excess enzyme conjugate, which is not specifically bound is washed away with wash buffer. A chromogenic substrate solution containing TMB is dispensed into the wells. After 15 minutes of incubation color development is stopped by adding the stop solution. The solution turns yellow at this point. The level of color is directly proportional to the concentration of IgG antibodies present in the original sample.

**3. REAGENTS, MATERIALS AND INSTRUMENTATION**

### 3.1. Reagents and materials supplied in the kit

<table>
<thead>
<tr>
<th>Reagents and materials supplied in the kit</th>
<th>REF</th>
<th>LOT</th>
<th>CAL0</th>
<th>CAL1</th>
<th>CAL2</th>
<th>CAL3</th>
<th>CAL4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti IFI16 Calibrators (5 vials, 1,2 mL each)</td>
<td>DCE002/12206-0</td>
<td>DCE002/12207-0</td>
<td>DCE002/12208-0</td>
<td>DCE002/12209-0</td>
<td>DCE002/12210-0</td>
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<tr>
<td>Phosphate buffer 0,1 M NaNO₃ &lt; 0.1%</td>
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<tr>
<td>Controls (2 vials, 1,2 mL each, ready to use)</td>
<td>DCE045/12201-0</td>
<td>DCE045/12202-0</td>
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<tr>
<td>Phosphate buffer 0,1 M NaNO₃ &lt; 0.1%</td>
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<tr>
<td>Sample diluent (1 vial, 100 mL)</td>
<td>DCE053-0</td>
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<td>Phosphate buffer 0,1 M NaNO₃ &lt; 0.1%</td>
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<tr>
<td>Conjugate (1 vial, 15 mL)</td>
<td>DCE002/12202-0</td>
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<tr>
<td>Anti h-IgG conjugated with horseradish peroxidase (HRP), BSA 0,1%, Proclin &lt; 0,0015%</td>
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<tr>
<td>Coated Microplate (1 breakable microplate)</td>
<td>DCE002/12203-0</td>
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<tr>
<td>Microplate coated with antigenic IFI16</td>
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<tr>
<td>TMB Substrate (1 vial, 15 mL)</td>
<td>DCE004-0</td>
<td></td>
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<tr>
<td>3,3',5,5'-tetramethylbenzidine 0,26 g/L, hydrogen peroxide 0,05%</td>
<td></td>
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<tr>
<td>Stop Solution (1 vial, 15 mL)</td>
<td>DCE005-0</td>
<td></td>
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<td></td>
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<tr>
<td>Sulphuric acid 0,15M</td>
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<tr>
<td>10X Conc. Wash Solution (1 vial, 50 mL)</td>
<td>DCE054-0</td>
<td></td>
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</tr>
<tr>
<td>Phosphate buffer 0,2M, proclin &lt; 0,0015%</td>
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</tbody>
</table>
3.2. Necessary Reagents that are not supplied
Distilled water.

3.3. Auxiliary materials and instrumentation
Automatic dispenser.

Microplate reader (450 nm)

4. WARNINGS

• This Anti IFI16 ELISA Assay Kit is intended for research use by professional persons only. Not for internal or external use in Humans or Animals.

• Use appropriate personal protective equipment while working with the reagents provided.

• Follow Good Laboratory Practice (GLP) for handling blood products.

• All human source material used in the preparation of the reagents has been tested and found negative for antibody to HIV 1&2, HbsAg, and HCV. No test method however can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, Calibrators and Controls should be handled in the same manner as potentially infectious material.

• Some reagents of the Anti IFI16 ELISA Assay Kit contain small amounts of Sodium Azide (NaN₃) or Proclin 300® as preservatives. Avoid the contact with skin or mucosa.

• Sodium Azide may be toxic if ingested or absorbed through the skin or eyes; moreover it may react with lead or copper plumbing to form potentially explosive metal azides. If you use a sink to remove the reagents, allow scroll through large amounts of water to prevent azide build-up.

• The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.

• The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.

• Avoid the exposure of reagent TMB/H₂O₂ to directed sunlight, metals or oxidants. Do not freeze the solution.

5. PRECAUTIONS

• Please adhere strictly to the sequence of pipetting steps provided in this protocol. The performance data represented here were obtained using specific reagents listed in this package insert.

• All reagents of the Anti IFI16 ELISA Assay Kit should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as indicated.

• Allow all Anti IFI16 ELISA Assay Kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.

• Do not interchange Anti IFI16 ELISA Assay Kit components from different lots. The expiry date printed on box and vials labels must be observed. Do not use any kit component beyond their expiry date.

• WARNING: the conjugate reagent is designed to ensure maximum dose sensitivity and may be contaminated by external agents if not used properly; therefore, it is recommended to use disposable consumables (tips, bottles, trays, etc.). For divided doses, take the exact amount of conjugate needed and do not re-introduce any waste product into the original bottle. In addition, for doses dispensed with the aid of automatic and semi-automatic devices, before using the conjugate, it is advisable to clean the fluid handling system, ensuring that the procedures of washing, deproteinization and decontamination are effective in avoiding contamination of the conjugate; this procedure is highly recommended when the kit is processed using analyzers which are not equipped with disposable tips.

• If you use automated equipment, the user has the responsibility to make sure that the kit has been appropriately tested.

• The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background.

• It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate.

• Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.

• Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera.

• Maximum precision is required for reconstitution and dispensation of the reagents.

• Samples microbiologically contaminated should not be used in the assay. Highly lipemic or haemolysed specimens should similarly not be used.

• Plate readers measure vertically. Do not touch the bottom of the wells.

6. PROCEDURE

6.1. Preparation of the Calibrators (C₀…C₆)

Since no international reference preparation for anti iFI16 antibodies is available, the assay system is calibrated in relative arbitrary units. The Calibrators are ready to use and have the following concentrations:

<table>
<thead>
<tr>
<th>AU/mL</th>
<th>C₀</th>
<th>C₁</th>
<th>C₂</th>
<th>C₃</th>
<th>C₄</th>
<th>C₅</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>50</td>
<td>100</td>
<td>200</td>
<td>800</td>
<td></td>
</tr>
</tbody>
</table>

Once opened, the Calibrators are stable 6 months at 2-8°C.

6.2. Preparation of the Sample

For determination of anti IFI16 human serum or plasma are the preferred sample matrices.

All serum and plasma samples have to be pre-diluted with sample diluent 1:100; for example, 10 μL of sample should be diluted with 990 μL of sample diluent. The samples need not to be fasting, and no special sample preparation is necessary. Collect blood by venipuncture into vacutainers and separate serum (after clot formation) or plasma from the cells by centrifugation.

Samples may be stored refrigerated at 2-8°C for at least 5 days. For longer storage of up to six months samples should be stored frozen at -20°C. To avoid repeated thawing and freezing the samples should be aliquoted.

Neither Bilirubin nor Hemolysis have significant effect on the procedure.

The Controls are ready to use.

6.3. Preparation of the Wash Solution

Dilute the contents of each vial of the buffered wash solution concentrate (10X) with distilled water to a final volume of 500 mL prior to use. For smaller volumes respect the 1:10 dilution ratio. The diluted wash solution is stable
for 30 days at 2-8°C. In concentrated wash solution it is possible to observe the presence of crystals. In this case mix at room temperature until complete dissolution of crystals is observed. For greater accuracy mix the whole vial of concentrated wash solution to 500 mL taking care to transfer the crystals, then mix until crystals are completely dissolved.

6.4. Procedure
- Allow all reagents to reach room temperature (22-28°C) for at least 30 minutes.
- Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C.
- To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.
- As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the calibration curve (C₀-C₄), two for each Control, two for each sample, one for Blank.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Calibrator</th>
<th>Sample/ Controls</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATTENTION:</td>
<td></td>
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<tr>
<td>in case of manual execution of the assay, measure the time required to dispense Calibrators and samples, and consider it to choose the appropriate length of first incubation time.</td>
<td></td>
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</tr>
<tr>
<td>Calibrator C₀-C₄</td>
<td>100 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>100 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diluted Sample</td>
<td>100 µL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For dispensing time of calibrators and samples less than 10 minutes incubate for 30 minutes at room temperature (22-28°C); alternatively, for dispensing time longer than 10 minutes incubate for 45 minutes at room temperature (22-28°C).

Remove the contents from each well, wash the wells three times with 300 µL diluted wash solution.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugate</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

Incubate for 30 minutes at room temperature (22-28°C). Remove the contents from each well, wash the wells three times with 300 µL diluted wash solution.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMB Substrate</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

Incubate for 15 minutes in the dark at room temperature (22-28°C).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stop Solution</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

Shake the microplate gently.
Read the absorbance (E) at 450 nm against Blank within 5 minutes.

7. RESULTS

7.1. Calculation of results
For Anti IFI16 a 4-Parameter-Fit with lin-log coordinates for optical density and concentration is the data reduction method of choice. Smoothed-Spline Approximation and log-log coordinates are also suitable. We recommend a Lin-Log Plot:
first calculate the averaged optical densities for each calibrator well. Use lin-log graph paper and plot the averaged optical density of each calibrator versus the concentration. Draw the best fitting curve approximating the path of all calibrator points. The calibrator points may also be connected with straight line segments. The concentration of unknowns may then be estimated from the calibration curve by interpolation.

8. REFERENCE VALUES
In a normal range study with serum samples from healthy blood donors the following ranges have been established with the Anti IFI16 test:

<table>
<thead>
<tr>
<th>Anti IFI16</th>
<th>Conc. (AU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>&lt; 80</td>
</tr>
<tr>
<td>Positive</td>
<td>&gt; 80</td>
</tr>
</tbody>
</table>

Please pay attention to the fact that the determination of a range of expected values for a “normal” population in a given method is dependent on many factors, such as specificity and sensitivity of the method used and type of population under investigation. Therefore each laboratory should consider the range given by the Manufacturer as a general indication and produce their own range of expected values based on the indigenous population where the laboratory works.

It is recommended that each laboratory establishes its own normal and pathological ranges of seric Anti IFI16.

9. PERFORMANCE AND CHARACTERISTICS

9.1. Specificity
99 healthy subjects have been tested in Eagle Biosciences Anti IFI16 assay; the specificity is 93.9%.

9.2. Sensitivity
237 samples affected by SSC (n°=147) and LES (n°=90) have been tested in Eagle Biosciences Anti IFI16 assay; total sensitivity is 36.7%.

9.3. Detection limit
The lowest concentration of anti IFI16 that can be distinguished from the Calibrator 0 is 1.16 AU/mL with a confidence limit of 95%.

9.4. Precision and reproducibility

9.4.1. Intra-Assay
Within run variation was determined by replicate 16 times the measurement of four different sera with values in the range of the calibration curve. The within assay variability is ≤ 4.2%.

9.4.2. Inter-Assay
Between run variation was determined by replicate measurements of two different control sera with different lots of kits and/or different mix of lots of reagents. The between assay variability is ≤ 5.8%.
10. WASTE MANAGEMENT
Reagents must be disposed off in accordance with local regulations.

BIBLIOGRAPHY
ERROR POSSIBLE CAUSES / SUGGESTIONS

No colorimetric reaction
- no conjugate pipetted reaction after addition
- contamination of conjugates and/or of substrate
- errors in performing the assay procedure (e.g. accidental pipetting of reagents in a wrong sequence or from the wrong vial, etc.)

Too low reaction (too low ODs)
- incorrect conjugate (e.g. not from original kit)
- incubation time too short, incubation temperature too low

Too high reaction (too high ODs)
- incorrect conjugate (e.g. not from original kit)
- incubation time too long, incubation temperature too high
- water quality for wash buffer insufficient (low grade of deionization)
- insufficient washing (conjugates not properly removed)

Unexplainable outliers
- contamination of pipettes, tips or containers
- insufficient washing (conjugates not properly removed) too high within-run
- reagents and/or strips not pre-warmed to CV% Room Temperature prior to use
- plate washer is not washing correctly (suggestion: clean washer head)
- too high between-run - incubation conditions not constant (time, CV % temperature)
- controls and samples not dispensed at the same time (with the same intervals) (check pipetting order)
- person-related variation