INTENDED USE
Immunoenzymatic colorimetric method for quantitative determination of IgE concentration in human serum or plasma.
IgE kit is intended for laboratory use only.

1. CLINICAL SIGNIFICANCE
Immunoglobulin E (IgE) is an antibody isotypes, found only in mammals. Although IgE is typically the least abundant isotype - blood serum IgE levels in a normal (“non-atopic”) individual are ~150ng/mL, compared to 10mg/mL for the IgGs (the isotypes responsible for most of the classical adaptive immune response) - it is capable of triggering the most powerful immune reactions. Most of our knowledge of IgE has come from an allergy known as type 1 hypersensitivity. IgE plays an important role in allergy, and in the immune system’s recognition of cancer.
People who suffer from true IgE-mediated allergies can have up to 10 times the normal level of IgE in their blood (as do sufferers of hyper-IgE syndrome). The IgE molecules (MW 200,000) bind to the surface of the mast cells and basophilic granulocytes. Subsequently the binding of allergen to cell-bound IgE causes these cells to release histamines and other vasoactive substances. The release of histamines in the body results initiates what is commonly known as an allergic reaction.
IgE levels show a slow increase during childhood, reaching adult levels in the second decade of life. In general, the total IgE levels increase with the allergies a person has and the number of times of exposure to the relevant allergens. Significant elevations may be seen in the sensitised individuals, but also in cases of myeloma, pulmonary aspergillosi, and during the active stages of parasitic infections.
The measurement of immunoglobulin E (IgE) in serum is widely used in the diagnosis of allergic reactions and parasitic infections. Before making any therapeutic determination it is important, however, to know whether the allergic reaction is IgE mediated or non-IgE mediated. Measurement of total IgE in serum sample, along with other supporting diagnostic information, can help to make that determination.

2. PRINCIPLE
The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme-linked and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-IgE antibody.
Upon mixing monoclonal biotinylated antibody, and a serum containing the native antigen, a reaction results between the native antigen and the antibody, forming an antibody-antigen complex. The interaction is illustrated by the following equation:

\[
\text{ka} \quad \text{Ag(IgE)} + \text{BtAb(m)} \leftrightarrow \text{Ag(IgE)} - \text{BtAb(m)}
\]

\[
\text{k-a} \quad \text{BtAb(m)} = \text{biotinylated monoclonal antibody (excess quantity)}
\text{Ag(IgE)} = \text{native antigen (variable quantity)}
\text{Ag(IgE)} - \text{BtAb(m)} = \text{antigen-antibody complex (variable quantity)}
\text{ka} = \text{rate constant of association}
\text{k-a} = \text{rate constant of dissociation}
\]

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

\[
\text{Ag(IgE)} - \text{BtAb(m)} + \text{Streptavidin C.W.} \rightarrow \text{Immobilized complex (IC)}
\]

\text{Streptavidin C.W.} = \text{Streptavidin immobilized on well}
\text{Immobilized complex (IC)} = \text{Ag-Ab bound to the well}

After a suitable incubation period, the antibody-antigen bound fraction is separated from unbound antigen by a washing step.
Another antibody (directed at a different epitope) labelled with the enzyme HRP (horseradish peroxidase) is added. Another interaction occurs to form an HRP-Antigen-Biotine complex on the surface of the wells, like in the following image:
Excess enzyme is washed off via a washing step. A suitable substrate (TMB) is added to produce colour measurable with the use of a microplate spectrophotometer. The enzyme activity on the well is directly proportional to the native free antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

### 3. REAGENTS, MATERIALS AND INSTRUMENTATION

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

1. **IgE Calibrators** (6 vials, 1 mL each)
   - CAL0
   - CAL1
   - CAL2
   - CAL3
   - CAL4
   - CAL5
   
2. **Anti IgE Biotin Conjugate** (1 vial, 13 mL)
   
3. **Anti IgE HRP Conjugate** (1 vial, 13 mL)
   
4. **Coated Microplate** (1 breakable microplate)
   
5. **TMB Substrate** (1 vial, 15 mL)
   
6. **Stop Solution** (1 vial, 15 mL)
   
7. **50X Conc. Wash Solution** (1 vial, 20 mL)

#### 3.2. Reagents necessary not supplied

- Distilled water.
- Automatic dispenser.
- Microplates reader (450 nm)

**Note**

Store the reagents at 2-8°C in the dark.

Open the bag of reagent 4 (Coated Microplate) only when it is at room temperature and close it immediately after use.

### 4. WARNINGS

- This kit is intended for in vitro 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, the Calibrators should be handled in the same manner as potentially infectious material.
- Some reagents contain small amounts of Proclin 300\textsuperscript{R} as preservative. Avoid the contact with skin or mucosa.
- 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/H\textsubscript{2}O\textsubscript{2} to directed sunlight, metals or oxidants. Do not freeze the solution.
- This method allows the quantitative determination of IgE from 5 IU/mL to 400 IU/mL.
- Serum IgE concentration is dependent upon a multiplicity of factors: including if the patient is sensitised, how many times the patient has been exposed to a specific allergen etc.
- Total IgE concentration alone is not sufficient to assess the clinical status. All the clinical findings especially specific allergy testing should be taken into consideration while determining the clinical status of the patient.
- Since all atopic reactions are not IgE mediated, all relevant clinical information should be taken into consideration before making any determination for patients who may be in the normal range.

### 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 Instruction For Use.
- All reagents 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 kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
- Do not interchange 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.
- 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 reagents.
• Samples microbiologically contaminated, highly
lipemic or haemolysed should not be used in the
assay.
• Plate readers measure vertically. Do not touch the
bottom of the wells.

6. PROCEDURE

6.1. Preparation of the Calibrators (C₀…C₅)
The Calibrators are standardized against the WHO
2nd IRP 75/502 and have the following concentrations:

<table>
<thead>
<tr>
<th>IU/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>5</td>
<td>25</td>
<td>50</td>
<td>150</td>
<td>400</td>
</tr>
</tbody>
</table>

Once opened, the Calibrators are stable 6 months at
2-8°C
For samples over 400 IU/mL dilute 1/50 with
Calibrator 0.

6.2. Preparation of Wash Solution
Dilute the content of each vial of the "50X Conc.
Wash Solution" with distilled water to a final volume of
1000 mL prior to use. For smaller volumes respect
the 1:50 dilution ratio. The diluted wash solution is
stable for 30 days at 2-8°C.

6.3. Preparation of the Sample
The usual precautions in the collection of
venipuncture samples should be observed.
For accurate comparison to established normal
values, a fasting morning serum sample should be
obtained.
To obtain the serum, the blood should be collected in
venipuncture tube without additives or anti-
coagulants; allow the blood to clot; centrifuge the
specimen to separate the serum from the cells.
Samples may be refrigerated at 2-8°C for a
maximum period of 48 hours. If the specimens cannot
be assayed within this time, they may be stored at
-20°C for up to 30 days. Avoid repetitive freezing and
thawing.
When assayed in duplicate, 0.050 mL of the
specimen is required.

6.4. Procedure
• Allow all reagents to reach room temperature
(22-28°C).
• 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-C₅), two for each Control, two
for each sample, one for Blank.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Calibrator</th>
<th>Sample</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator</td>
<td>C₀-C₅</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td></td>
<td>25 µL</td>
<td></td>
</tr>
<tr>
<td>Anti IgE Bi</td>
<td>100 µL</td>
<td>100 µL</td>
<td></td>
</tr>
<tr>
<td>anti IgE Conjugate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti IgE HRP Concentrate</td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>TMB Substrate</td>
<td></td>
<td>100 µL</td>
<td></td>
</tr>
<tr>
<td>Stop Solution</td>
<td></td>
<td>100 µL</td>
<td></td>
</tr>
</tbody>
</table>

Incubate at room temperature (22-28°C) for 30
minutes. Remove the contents from each well. Wash the wells
3 times with 0.3 mL of diluted washing solution.

7. QUALITY CONTROL
Each laboratory should assay controls at levels in the
low, medium and high range for monitoring assay
performance. These controls should be treated as
unknowns and values determined in every test
procedure performed. Quality control charts should be
maintained to follow the performance of the supplied
reagents. Pertinent statistical methods should be
employed to ascertain trends. Significant deviation
from established performance can indicate unnoticed
change in experimental conditions or degradation of
kit reagents. Fresh reagents should be used to
determine the reason for the variations.
8. RESULTS

8.1. Note
Maximum Absorbance (CAL. 5) = >1.0
Optical densities (O.D.) higher than 2.0 could be out of the measurement range of some microplate readers. It is therefore necessary, for O.D.s higher than 2.0, to perform a reading at 405 nm (=wavelength of peak shoulder) in addition to 450 nm (peak wavelength) and 620 (reference filter for the subtraction of interferences due to the plastic).
For microplate readers unable to read the plate at 3 wavelengths at the same time, it is advisable to proceed as follows:
- Read the microplate at 450 nm and at 620 nm.
- Read again the plate at 405 nm and 620 nm.
- Find out the wells whose ODs at 450 nm are higher than 2.0
- Select the corresponding ODs read at 405 nm and multiply these values at 405 nm by the conversion factor 3.0 (where OD 450/OD 405 = 3.0), that is: OD 450 nm = OD 405 nm x 3.0
Warning: The conversion factor 3.0 is suggested only. For better accuracy, the user is advised to calculate the conversion factor specific for his reader.

8.2. Mean Absorbance
Calculate the mean of the absorbance (Em) for each point of the standard curve and of each sample.

8.3. Standard Curve – Automatic method
Use the 4 parameters logistic – preferred – or the smoothed cubic spline function as calculation algorithm.
If computer controlled data reduction is used to calculate the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.

A dose response curve is used to ascertain the concentration of IgE in unknown specimens.
Record the absorbance obtained from the printout of the microplate reader.
Plot the absorbance for each duplicate serum reference versus the corresponding IgE concentration in IU/mL on linear graph paper
Draw the best-fit curve through the plotted points.
To determine the concentration of IgE for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in IU/mL) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated).

9. REFERENCE VALUES

<table>
<thead>
<tr>
<th>Age</th>
<th>Median IU/mL</th>
<th>Range IU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 – 3</td>
<td>6.4</td>
<td>0 – 46</td>
</tr>
<tr>
<td>3 – 16</td>
<td>25.0</td>
<td>0 – 280</td>
</tr>
<tr>
<td>Adults</td>
<td>43</td>
<td>0 – 200</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.

10. PERFORMANCE AND CHARACTERISTICS

10.1. Precision

10.1.1. Intra Assay Variation
Within run variation was determined by replicate measurements (16x) of three different control sera in one assay. The within assay variability is ≤ 7.2%.

10.1.2. Inter Assay Variation
Between run variations was determined by replicate measurements (16x) of three different control sera in different lots of kits. The between assay variability is ≤ 7.6%.

10.2. Accuracy
The recovery has been performed by adding 50 – 100 – 200 IU/mL of IgE to three samples. The results are reported in Table:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Measured</th>
<th>Recovered</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool1</td>
<td>10.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pool1 + 50</td>
<td>61.3</td>
<td>50.7</td>
<td>101.4</td>
</tr>
<tr>
<td>Pool1 + 100</td>
<td>116.2</td>
<td>105.6</td>
<td>105.6</td>
</tr>
<tr>
<td>Pool1 + 200</td>
<td>209.1</td>
<td>1985</td>
<td>99.3</td>
</tr>
<tr>
<td>Pool2</td>
<td>65.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pool2 + 50</td>
<td>112.3</td>
<td>46.5</td>
<td>93.0</td>
</tr>
<tr>
<td>Pool2 + 100</td>
<td>165.6</td>
<td>99.8</td>
<td>99.8</td>
</tr>
<tr>
<td>Pool2 + 200</td>
<td>258.1</td>
<td>192.3</td>
<td>96.2</td>
</tr>
<tr>
<td>Pool3</td>
<td>25.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pool3 + 50</td>
<td>76.3</td>
<td>51.0</td>
<td>102.0</td>
</tr>
<tr>
<td>Pool3 + 100</td>
<td>122.5</td>
<td>97.2</td>
<td>97.2</td>
</tr>
<tr>
<td>Pool3 + 200</td>
<td>225.2</td>
<td>199.9</td>
<td>100.0</td>
</tr>
</tbody>
</table>

10.3. Sensitivity
The lowest detectable concentration of IgE that can be distinguished from the zero Calibrator is 0.27 IU/mL at the 95 % confidence limit.

10.4. Specificity
In order to assess the specificity of the antibody pair used for the IgE Elisa assay, massive doses of related analytes were spiked in a pool of patient sera:

<table>
<thead>
<tr>
<th>Cross Reagent</th>
<th>U.M.</th>
<th>Tested Concentration</th>
<th>Cross reactivity</th>
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<tbody>
<tr>
<td>IgE</td>
<td>IU/mL</td>
<td>---</td>
<td>100%</td>
</tr>
<tr>
<td>IgA</td>
<td>IU/mL</td>
<td>1000</td>
<td>None Detected</td>
</tr>
<tr>
<td>IgM</td>
<td>IU/mL</td>
<td>1000</td>
<td>None Detected</td>
</tr>
<tr>
<td>IgG</td>
<td>IU/mL</td>
<td>1000</td>
<td>None Detected</td>
</tr>
</tbody>
</table>

According to the data the antibody pair was found to be highly specific for the IgE only.
10.5. Correlation
Diametra IgE ELISA was compared to another commercially available IgE assay. 214 serum samples were analysed according in both test systems.
The linear regression curve was calculated:
\[ y = 1.175 \times - 11.172 \]
\[ r^2 = 0.972 \]
y = IgE commercial kit
x = IgE Diametra kit

11. WASTE MANAGEMENT
Reagents must be disposed off in accordance with local regulations.

BIBLIOGRAPHY
- National Committee for Clinical laboratory Standards:
  - Procedures for the collection of blood specimens by
- Tietz NW, Clinical Guide to Laboratory Tests, 3rd.
<table>
<thead>
<tr>
<th>DE</th>
<th>In vitro Diagnostikum</th>
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<tr>
<td>ES</td>
<td>Producto sanitario para diagnóstico In vitro</td>
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<td>ES</td>
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<td>ES</td>
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<td>Consulter le mode d’emploi</td>
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<td>ES</td>
<td>Número de catálogo</td>
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<td>FR</td>
<td>Tenir à l'ecart de la lumière du soleil</td>
<td>FR</td>
<td>Références du catalogue</td>
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<td>GB</td>
<td>Keep away from sunlight</td>
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<td>IT</td>
<td>Tenere lontano dalla luce solare</td>
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<td>PT</td>
<td>Mantenha longe da luz solar</td>
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SUGGERIMENTI PER LA RISOLUZIONE DEI PROBLEMI/TROUBLESHOOTING

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