IgE

Enzyme immunoassay for the quantitative determination of IgE in human serum

Only for in-vitro diagnostic use

Product Number: DNOV102 (96 Determinations)
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1. INTRODUCTION

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 vasoattive 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 aspergillosis, 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. INTENDED USE

Immunoenzymatic colorimetric method (ELISA) for quantitative determination of IgE in serum.

3. PRINCIPLE OF THE ASSAY

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme 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, reaction results between the native antigen and the antibody, forming an Antibody-Antigen complex. The interaction is illustrated by the following equation:

\[
\begin{align*}
\text{Ag(IgE)} + \text{BtnAb (m)} & \xrightarrow{\text{ka}} \text{Ag(IgE)-BtnAb(m)} \\
\text{Ag(IgE)-BtnAb (m) + Streptavidin CW} & \xrightarrow{\text{k-a}} \text{Immobilized Complex (IC)}
\end{align*}
\]

BtnAb(m) = Biotinylated Monoclonal Antibody (Excess Quantity)
AgIgE = Native Antigen (Variable Quantity)
Ag(IgE)-BtnAb(m) = Antigen-Antibodies Complex (variable quantity)
ka = Rate Constant of Association
k-a = 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:

\[
\begin{align*}
\text{Ag(IgE)-BtnAb (m)+Streptavidin CW} & \xrightarrow{\text{Immobilized Complex (IC)}}
\end{align*}
\]

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

After a suitable incubation period, the antibody-antigen bound fraction is separated from unbound antigen by decantation or aspiration.

Another antibody (directed at a different epitope) labelled with an enzyme is added. Another interaction occurs to form an Enzyme labelled Antibody-Antigen-Biotinylated-Antibody complex on the surface of the wells. Excess enzyme is washed off via a wash step. A suitable substrate 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.
kb

\[ IC + E(\text{Ab-IgE}) \rightleftharpoons E(\text{Ab-IgE})-IC \]

\[ k_b \]

\( E(\text{Ab-IgE}) = \) Enzyme labelled Antibody (excess quantity)

\( E(\text{Ab-IgE})-\text{IC} = \) Antigen-Antibodies complex

\( kb = \) Rate Constant of Association

\( k_b = \) Rate constant of dissociation

4. MATERIALS

4.1. Reagents supplied

- **Coated Wells**: 12 breakapart 8-well snap-off strips coated with streptavidin, in resealable aluminium foil.
- **Stop Solution**: 1 bottle containing 15 ml sulphuric acid, 0.15 mol/l (avoid any skin contact).
- **IgE Biotin conjugate**: 1 bottle containing 13 ml of biotinylated IgE.
- **Enzyme conjugate**: 1 bottle containing 13 ml of anti-human IgE-HRP conjugate
- **TMB Substrate Solution**: 1 bottle containing 15 ml 3,3’,5,5’-tetramethylbenzidine (H₂O₂-TMB 0.25g/l) (avoid any skin contact).
- **Wash solution 50x conc.**: 1 bottle containing 20 ml NaCl (45g/l) and Tween20 (55 g/l)
- **IgE Standards**: 6 bottles, 1 ml each. The standards are calibrated against the (WHO 2nd IRP 75/502) and have the following concentrations:
  - Standard 0: 0 IU/ml
  - Standard 1: 5 IU/ml
  - Standard 2: 25 IU/ml
  - Standard 3: 50 IU/ml
  - Standard 4: 150 IU/ml
  - Standard 5: 400 IU/ml

4.2. Materials supplied

- 1 Strip holder
- 1 Cover foils
- 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, 405 and 620 nm
- Manual or automatic equipment for rinsing wells
- Pipettes (precision better than 1.5%)
- Vortex tube mixer
- Distilled water
- Disposable tubes
- Timer

5. STABILITY AND STORAGE

The closed reagents are stable up to the expiry date stated on the label when stored at 2…8 °C.

Opened reagents are stable for 60 days when stored at 2…8°C.

6. REAGENT PREPARATION

*It is very important to bring all reagents, samples and standards to room temperature (22…28°C) before starting the test run!*

6.1. Coated snap-off Strips

The ready to use break apart snap-off strips are coated streptavidin. 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 expiry date. Do not remove the adhesive sheets on the unused strips.**

6.2. IgE Biotin Conjugate

The bottle contains 13 ml of a ready-to-use conjugate.
6.3 Enzyme Conjugate
The bottle contains 13 ml of a ready-to-use conjugate.

6.4. Standards
Each of the 6 vials contains 1 ml standard solution of the concentration mentioned in 4.1. The standards are ready to use.

6.5. 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 in the dark. 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 use the TMB substrate solution is still stable for another 6 months if stored at 2...8°C.

6.6. Stop Solution
The bottle contains 15 ml 0.15 M sulphuric acid solution (R 36/38, S 26). This ready to use solution has to be stored at 2...8°C. After first use stable until expiry date.

6.7. Wash Solution
Dilute the 50x concentrated wash solution to 1000 ml with distilled water in a suitable storage container. For smaller volumes respect the 1:50 dilution ratio. The diluted wash solution is stable for 30 days at 2…8°C.

7. SPECIMEN COLLECTION AND PREPARATION
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 specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050 ml of the specimen is required. Samples with an IgE concentration over 400 IU/ml should be diluted 1:50 with standard 0.

7.1. Precaution
- Avoid the exposure of TMB substrate to direct sunlight, metal or oxidants.
- Maximum precision is required for dispensation of the reagents.
- This method allows the determination of IgE from 5 to 400 IU/ml.

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. 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. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than one plate is used, it is recommended to repeat the dose response curve. Please allocate at least:

- 1 well (e.g. A1) for the substrate blank
- 2 wells (e.g. B1+C1) for standard 0
- 2 wells (e.g. D1+E1) for standard 1
- 2 wells (e.g. F1+G1) for standard 2
- 2 wells (e.g. H1+A2) for standard 3
- 2 wells (e.g. B2+C2) for standard 4
- 2 wells (e.g. D2+E2) for standard 5

It is necessary to determine standards 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 and each patient sample.

1. Dispense 25 µl standards and samples into their respective wells. Add 100 µl IgE biotin conjugate to each well. Leave well A1 for substrate blank.
2. Cover wells with the foil supplied in the kit.
3. Incubate for 30 min at room temperature (22...28°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 diluted wash solution. Avoid overflows from the reaction wells. Leave the excess liquid to drain away by inverting the plate on absorbent paper!

Note: Washing is critical! Insufficient washing results in poor precision and falsely elevated absorbance values.
5. Dispense 100 µl Enzyme conjugate into all wells except blank.

6. **Incubate for exactly 30 min at room temperature (22…28°C).**

7. When incubation has been completed, remove the foil, aspirate the content of the wells and wash each well three times with 300 µl diluted wash solution. Avoid overflows from the reaction wells. Leave the excess liquid to drain away by inverting the plate on absorbent paper!

8. Dispense 100 µl TMB solution into all wells.

9. **Incubate for exactly 15 min at room temperature (22…28°C) in the dark.**

10. Dispense 100 µl Stop Solution into all wells in the same order and at the same rate as for the TMB Substrate Solution. Shake the microplate gently. **Any blue colour developed during the incubation turns into yellow.**

11. Measure the absorbance of the specimen at 450 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 standard and patient sample in the distribution and identification plan.

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

### 9. RESULTS

#### 9.1. Validity of the assay

Maximum absorbance of standard 5 ≥ 1.0

#### 9.2. OD Conversion

The optical densities (O.D.) of some standards and samples may be higher than 2.0, in such a case, they could be out of the measurement range of the microplate reader. 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 own reader.

#### 9.3. Calculation

**Automated method**

Use the 4 parameter logistic (preferred) or the smoothed cubic spline function as calculation algorithm.

**Manual method**

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).

#### 9.4. Reference Values

<table>
<thead>
<tr>
<th>Age</th>
<th>Median</th>
<th>Range</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>
10. 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.

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.

11. SPECIFIC PERFORMANCE CHARACTERISTICS

11.1. Precision

Intra Assay Variation

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

Inter Assay Variation

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

11.2. 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>
</tr>
</thead>
<tbody>
<tr>
<td>IgE IU/mL</td>
<td>---</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>IgA IU/mL</td>
<td>1000</td>
<td>None Detected</td>
<td></td>
</tr>
<tr>
<td>IgM IU/mL</td>
<td>1000</td>
<td>None Detected</td>
<td></td>
</tr>
<tr>
<td>IgG IU/mL</td>
<td>1000</td>
<td>None Detected</td>
<td></td>
</tr>
</tbody>
</table>

According to the data the antibody pair was found to be highly specific for the IgE only.

11.3. Sensitivity

The lowest detectable concentration of IgE that can be distinguished from standard 0 is 0.27 IU/ml at the 95% confidence limit.

11.4. Accuracy

The recovery has been performed by adding 50 – 100 – 200 IU/ml of IgE to three samples. The results are reported in the table:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Measured IU/ml</th>
<th>Recovered IU/ml</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>81.4</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>
11.5. Correlation

The NovaTec 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

\[ y = 1.175 x - 11.172 \]

\[ r^2 = 0.972 \]

\( y = \) IgE Predicate kit
\( x = \) IgE NovaTec kit

12. LIMITATIONS OF THE PROCEDURE

Sample(s), which are contaminated microbiologically, should not be used in the assay. Highly lipemic or haemolysed specimen(s) should similarly not be used. 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 (10) minutes to avoid assay drift. If more than one (1) plate is used, it is recommended to repeat the dose response curve. Addition of the substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the addition of the substrate and the stopping solution should be added in the same sequence to eliminate any time deviation during reaction. Plate readers measure vertically. Do not touch the bottom of the wells. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.

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.

13. PRECAUTIONS AND WARNINGS

- In compliance with article 1 paragraph 2b European directive 98/79/EC the use of the in vitro diagnostic medical devices is intended by the manufacturer to secure suitability, performances and safety of the product. Therefore the test procedure, the information, the precautions and warnings in the instructions for use have to be strictly followed. The use of the testkits with analyzers and similar equipment has to be validated. Any change in design, composition and test procedure as well as for any use in combination with other products not approved by the manufacturer is not authorized; the user himself is responsible for such changes. The manufacturer is not liable for false results and incidents for these reasons. The manufacturer is not liable for any results by visual analysis of the patient samples.
- Only for in-vitro diagnostic 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.

**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!

13.1. 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.
13. LITERATURE

National Committee for Clinical laboratory Standards:

14. ORDERING INFORMATION

Prod. No.: DNOV102 IgE Determination (96 Determinations)
SCHEME OF THE ASSAY

IgE

Test Preparation

Prepare reagents and samples as described.
Establish the distribution and identification plan for all specimens and controls on the result sheet 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>Stand. 0</th>
<th>Stand. 1</th>
<th>Stand. 2</th>
<th>Stand. 3</th>
<th>Stand. 4</th>
<th>Stand. 5</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stand. 0</td>
<td>-</td>
<td>25 µl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stand. 1</td>
<td>-</td>
<td>-</td>
<td>25 µl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stand. 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25 µl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stand. 3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25 µl</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stand. 4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stand. 5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25 µl</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25 µl</td>
</tr>
<tr>
<td>IgE biotin Conjugate</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

Cover wells with foil supplied in the kit
Incubate for 30 min at room temperature (22…28°C)
Wash each well three times with 300 µl diluted Wash Solution

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Stand. 0</th>
<th>Stand. 1</th>
<th>Stand. 2</th>
<th>Stand. 3</th>
<th>Stand. 4</th>
<th>Stand. 5</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme conjugate</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
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</table>

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<th>Stand. 2</th>
<th>Stand. 3</th>
<th>Stand. 4</th>
<th>Stand. 5</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMB Substrate</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

Incubate for exactly 15 min at room temperature (22…28°C) in the dark

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Stand. 0</th>
<th>Stand. 1</th>
<th>Stand. 2</th>
<th>Stand. 3</th>
<th>Stand. 4</th>
<th>Stand. 5</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stop Solution</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

Shake the microplate gently.
Photometric measurement at 450 nm

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