Thyroglobulin

Enzyme immunoassay for the quantitative determination of Thyroglobulin in human serum

Only for in-vitro diagnostic use

Product Number: DNOV057 (96 Determinations)
1. INTRODUCTION

Thyroglobulin (Tg), a glycoprotein with a molecular weight of about 660,000 Daltons, is the thyroid’s main iodine protein and the most important compound of follicular colloid. Thyroglobulin is the form under which the active hormones T3 and T4 together with their immediate forerunners MIT and DIT are laid inside the thyroid gland. The clinical applications of the hTg dosage seem to originate from its specificity for the thyroid and related cells.

The dosage of hTg can be used as support to scintigraphies or other techniques for studying pathogenesis, making a diagnosis and analyzing the course of thyroid disorders.

The dosage of Tg before and after replacement treatment with L-Thyroxin cannot be established in cases of hypothyroidism due to thyroid agenesis. In cases of secondary hypothyroidism with a dysglandular goiter or ectopic thyroid, the levels of hTg are normal or high. The circulating levels of hTg tend to increase in several thyroid disorders such as toxic and atoxic goiter, subacute thyroiditis, Basedow’s disease and carcinoma. In Basedow’s disease the hTg dosage is a potentially interesting index of normalization of hyperthyroidism in patients treated with anti-thyroid drugs. In the oncology field and more specifically for differentiated thyroid carcinoma, there are very promising applications linked to the ability of thyroid tumors tissues to concentrate iodine and synthesize hTg as a normal thyroid. Basically the dosage of hTg can be used as follows:

a. Pre-operating diagnosis of thyroid tumors.
   This application does not allow the differentiated diagnosis of the tumor as the values of hTg seen in malignant and benign nodules are superimposable.

b. Post-operation monitoring
   In patients treated surgically or with radiotherapy, long lasting hTg levels suggest the presence of a residual carcinoma and/or carcinoma with metastasis.

c. Monitoring of totally thyroidectomized patients
   The use of circulating hTg as an indicator of recurrent tumors (metastasis marker) has an established clinical value: the increase of Thyroglobulinaemia indicates the need to undergo further analysis for confirming the diagnosis. Interesting advantages can come from: a) a reduced use of scintigraphic diagnostic techniques as they imply regular suspension of replacement treatment and frequent exposure to radiation, b) and complete completion of the information obtained via scintigraphy.

2. INTENDED USE

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

3. PRINCIPLE OF THE ASSAY

Four different anti-Thyroglobulin monoclonal antibodies are used. Three antibodies are coated on the wells; the fourth is soluble and conjugated to Biotin. Thyroglobulin in samples and standards binds to the immobilised antibodies on the surface of the microtiter wells and the second, soluble anti-Thyroglobulin antibody conjugated with biotin binds to the immobile antibody-Thyroglobulin-complex during the first incubation. After a washing step Streptavidin conjugated with horseradish peroxidase (HRP) is added to all wells and reacts with the biotinylated antibodies. A second washing step is performed. Then the immune complex is visualized by adding Tetramethylbenzidine (TMB) substrate, which gives a blue reaction product. The intensity of this product is proportional to the amount of Thyroglobulin in samples and standards. Sulphuric acid is added to stop the reaction. This produces a yellow endpoint colour. Absorption at 450 and 405 nm is read using an ELISA microwell plate reader.

4. MATERIALS

4.1. Reagents supplied

- Anti-Thyroglobulin Coated Wells: 12 breakapart 8-well snap-off strips coated with three different anti-Thyroglobulin antibodies; in resealable aluminium foil.
- Stop Solution: 1 bottle containing 12 ml sulphuric acid, 0.15 mol/l (avoid any skin contact).
- Anti-Thyroglobulin - Biotin Conjugate: 1 bottle containing 13 ml of biotin labelled anti-Thyroglobulin antibodies.
- Streptavidine-HRP Conjugate: 1 bottle containing 15 ml horseradish peroxidase labelled Streptavidine.
- Recovery Solution: 1 bottle containing 3 ml of Thyroglobulin in proteic matrix (50 ng/ml).
- TMB Substrate Solution: 1 bottle containing 12 ml 3, 3´, 5, 5´-tetramethylbenzidine (H2O2-TMB 0.26 g/l) (avoid any skin contact).
- Wash Solution 20x conc.: 1 bottle containing 50 ml (NaCl 9 g/l, Tween20 22 g/l)
- Control Serum I: 1 bottle containing 1 ml Thyroglobulin in proteic matrix.
- Control Serum II: 1 bottle containing 1 ml Thyroglobulin in proteic matrix.
Thyroglobulin Standards: 7 bottles, 2 ml of standard 0, 1 ml each of all other standards. The standards have the following concentrations:

- Standard 0: 0 ng/ml
- Standard 1: 1 ng/ml
- Standard 2: 3 ng/ml
- Standard 3: 10 ng/ml
- Standard 4: 30 ng/ml
- Standard 5: 100 ng/ml
- Standard 6: 300 ng/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 405, 450 and 620 nm
- Manual or automatic equipment for rinsing wells
- Absorbent paper for blotting the microplate wells
- Pipettes to deliver 50 µl volumes with a precision better than 1.5%
- Dispenser for repetitive deliveries of 100 and 300 µl with a precision better than 1.5%
- Vortex tube mixer
- 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. After opening 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 with anti-Thyroglobulin antibodies. 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. Anti-Thyroglobulin-Biotin Conjugate

The bottle contains 13 ml of a ready to use solution with anti-Thyroglobulin antibodies conjugated with biotin.

6.3. Streptavidine-HRP Conjugate

The bottle contains 15 ml of a ready to use solution with Streptavidine conjugated with HRP.

6.4. Recovery Solution

The bottle contains 3 ml of a ready to use solution.

6.5. Standards

The standards (human serum reference) are ready to use. A preservative has been added.

6.6. TMB Substrate Solution

The bottle contains 12 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.

6.7. Stop Solution

The bottle contains a ready to use solution of 0.15 M sulphuric acid solution (R 36/38, S 26).

6.8. Wash Solution

Dilute the concentrated Wash Solution with distilled water to 1000 ml. For smaller volumes respect the 1:20 dilution ratio. Once diluted it is stable for 30 days at 2…8°C.
6.9. Control Serum

Control serum I and II are ready to use.

7. SPECIMEN COLLECTION AND PREPARATION

The assay can be performed in serum samples. Highly lipemic or haemolysed samples must be discarded. Keep samples at 2...8°C for 1-2 days; for longer periods it is advisable to freeze samples at -20°C. Repeated freezing and thawing of samples should be avoided. Samples with Tg concentrations higher than 300 ng/ml, must be diluted with standard 0. We suggest a dilution of 1:5 (100 µl of sample + 400 µl of standard 0).

7.1. Warnings and Precautions

− Do not store or leave reagents and samples at high temperatures or areas of possible contamination.
− Use thoroughly clean glassware, free from metal ion contamination or oxidizing substances.
− Use distilled or deionized water, stored in perfectly clean containers.
− Carefully avoid any contamination among samples; for this purpose, disposable tips should be used for each sample and reagent.
− Do not modify in any way the "Assay Procedure". If you do not respect exact incubation times, temperature and quantities of added reagent, incorrect clinical results may occur.
− Reconstitute lyophilized reagents, if present, as described on the relative labels. Any deviation in reagent use or wrong volumes, may affect the reliability of results obtained.
− In case of manual procedure, it is important to use calibrated pipettes and have appropriate technical manuals. Primary importance is a good precision preparing and dispensing the reagents. Ensure that all the equipment used is in perfect working order, has been correctly calibrated and is regularly maintained.
− Ensure that all the equipment used (glassware, dry heater, microplate shaker, microplate washers, spectrophotometer and fridge/freezers used for reagent and sample storage) is in perfect working order, has been correctly calibrated and is regularly maintained. Any deviation from the correct use of the equipment listed can produce errors in the methodology; this may affect the reproducibility and reliability of results obtained.
− Utilise a suitable method for the correct identification of patient samples. Incorrect identification may cause specificity losses of the system and wrong clinical results.

In order to avoid personal and environmental contamination, the following precautions must be observed:

− Use disposable gloves while handling potentially infectious material and while performing the assay.
− Do not pipette reagents by mouth.
− Do not smoke, eat, drink or apply cosmetics during the assay.
− TMB substrate solution and Stop solution should be handled with care. Avoid contact with skin, eyes and mucous membranes. In case of accident rinse thoroughly with running water.
− Avoid splashing and aerosol formation; in such cases, carefully wash with a 3% sodium hypochlorite solution. Any such cleaning material must be treated as potentially infectious and disposed of accordingly.
− Some reagents contain sodium azide as preservative; to prevent build-up of explosive metal azides in lead and copper plumbing, reagents should be discarded by flushing the drain with large amounts of water.

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. Pay attention to 10.6. Prior to commencing the assay, the distribution and identification plan for all specimens, controls 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 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. 1+A2) | for standard 3 |
| 2 wells (e.g. B2+C2) | for standard 4 |
| 2 wells (e.g. D2+E2) | for standard 5 |
| 2 wells (e.g. F2+G2) | for standard 6 |
| 2 wells (e.g. H2+A3) | for control serum I |
| 2 wells (e.g. B3+C3) | for control serum II |

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 and each patient sample.

1. Dispense 50 µl standards, controls and samples into their respective wells. Leave well A1 for substrate blank. Add 100 µl Anti-Thyroglobulin-Biotin conjugate to each standard, control and sample.
2. Cover wells with the foil supplied in the kit.
3. Incubate for 1.5 hour at 37 °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. The soak time between each wash cycle should be >5sec. 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.
5. Dispense 100 µl Streptavidine-HRP-Conjugate into all wells and cover them with the foil supplied in the kit.
6. Incubate for 30 min at 37 °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. The soak time between each wash cycle should be >5sec. At the end carefully remove remaining fluid by tapping strips on tissue paper prior to the next step!
8. Dispense 100 µl TMB Substrate 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 after zeroing the instrument with standard 0.

9. RESULTS

9.1. Calculation of Results
In order to obtain a better sensitivity, the present method employs spectrophotometric reading at two wavelengths (450 and 405 nm). For samples with Thyroglobulin concentrations ranging from 0 to 30 ng/ml, read at 450 nm wavelength; for samples with Thyroglobulin level higher than 30 ng/ml, read at 405 nm wavelength. Draw a standard curve on millimetric graph paper, by plotting the standard concentrations (x-axis) against the absorbance obtained for each standard (y-axis). Corresponding Thyroglobulin concentrations in ng/ml are obtained by interpolating the absorbencies of each sample on the calibration curve; in case of diluted samples, multiply by the dilution factor.

9.2 Example of Calculation
The values shown below must be considered as an example and must not be used in place of experimental data.

<table>
<thead>
<tr>
<th>Standard/Sample</th>
<th>O.D. 450 nm</th>
<th>Thyro-globulin O.D. 405 nm</th>
<th>Thyro-globulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard 0 ng/ml</td>
<td>0.020</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>Standard 1 ng/ml</td>
<td>0.047</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td>Standard 3 ng/ml</td>
<td>0.127</td>
<td>0.042</td>
<td></td>
</tr>
<tr>
<td>Standard 10 ng/ml</td>
<td>0.313</td>
<td>0.098</td>
<td></td>
</tr>
<tr>
<td>Standard 30 ng/ml</td>
<td>0.863</td>
<td>0.373</td>
<td></td>
</tr>
<tr>
<td>Standard 100 ng/ml</td>
<td>1.650</td>
<td>0.603</td>
<td></td>
</tr>
<tr>
<td>Standard 300 ng/ml</td>
<td>&gt; 3.000</td>
<td>1.488</td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>0.450</td>
<td>15.4 ng/ml</td>
<td>0.141</td>
</tr>
<tr>
<td>Sample 2</td>
<td>2.240</td>
<td>0.829</td>
<td>166.4 ng/ml</td>
</tr>
</tbody>
</table>

9.3 Normal Values
The value reported below is indicative. We suggest that each laboratory establishes its own normal range. Normal values have been established on 85 healthy subjects without thyroid disorders. Thyroglobulin levels were lower than 40 ng/ml.

9.4 Validation Criteria
Before proceeding to calculate the results, make sure that the control serum concentration is within the value described on the certificate of analysis.

10. SPECIFIC PERFORMANCE CHARACTERISTICS

10.1 Specificity
No cross reaction was observed with MIT, DIT, rT3, T3, T4, TSH, FSH and LH. This analytic method showed a cross-reactivity of 0.01% with human TBG.
10.2 Sensitivity
The sensitivity was calculated upon the standard curve and expressed as the minimal dose showing a significant difference from standard 0 (mean value + 2 S.D.). This dose is 0.15 ng/ml.

10.3 Precision
Precision was evaluated determining the repeatability and the reproducibility of the assay (intra- and inter-assay variability), on 3 sera at different Thyroglobulin concentrations.

**Intra-assay**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Media (ng/ml) ± SD</th>
<th>% C.V.</th>
<th>Replicates n.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.69 ± 0.10</td>
<td>6.14</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>15.14 ± 0.26</td>
<td>1.71</td>
<td>10</td>
</tr>
<tr>
<td>C</td>
<td>152.41 ± 5.71</td>
<td>3.75</td>
<td>10</td>
</tr>
</tbody>
</table>

**Inter-assay**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Media (ng/ml) ± SD</th>
<th>% C.V.</th>
<th>Replicates n.</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>1.8 ± 0.13</td>
<td>6.9</td>
<td>10</td>
</tr>
<tr>
<td>b</td>
<td>38.1 ± 3.0</td>
<td>7.8</td>
<td>10</td>
</tr>
<tr>
<td>c</td>
<td>150.4 ± 9.30</td>
<td>6.2</td>
<td>10</td>
</tr>
</tbody>
</table>

10.4. Accuracy
Accuracy of the method has been checked by the recovery and parallelism tests:

**Recovery Test.**
Known amounts of Thyroglobulin have been added to two normal sera and tested.

<table>
<thead>
<tr>
<th>Added (ng/ml)</th>
<th>Expected (ng/ml)</th>
<th>Recovered (ng/ml)</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 -</td>
<td>-</td>
<td>1.9</td>
<td>-</td>
</tr>
<tr>
<td>S1 + 12.5</td>
<td>13.0</td>
<td>14.9</td>
<td>104.0</td>
</tr>
<tr>
<td>S1 + 25</td>
<td>23.3</td>
<td>25.2</td>
<td>93.2</td>
</tr>
<tr>
<td>S1 + 50</td>
<td>48.8</td>
<td>50.7</td>
<td>97.6</td>
</tr>
<tr>
<td>S1 + 100</td>
<td>91.7</td>
<td>93.6</td>
<td>91.7</td>
</tr>
<tr>
<td>S1 + 200</td>
<td>193.1</td>
<td>195.00</td>
<td>96.6</td>
</tr>
<tr>
<td>S2 -</td>
<td>17.4</td>
<td>17.4</td>
<td>-</td>
</tr>
<tr>
<td>S2 + 12.5</td>
<td>9.6</td>
<td>27.0</td>
<td>76.8</td>
</tr>
<tr>
<td>S2 + 25</td>
<td>24.6</td>
<td>42.0</td>
<td>98.4</td>
</tr>
<tr>
<td>S2 + 50</td>
<td>47.6</td>
<td>65.0</td>
<td>95.2</td>
</tr>
<tr>
<td>S2 + 100</td>
<td>98.8</td>
<td>116.2</td>
<td>98.8</td>
</tr>
<tr>
<td>S2 + 200</td>
<td>187.7</td>
<td>205.1</td>
<td>93.9</td>
</tr>
</tbody>
</table>

**Parallelism Test**
Two patient sera with high Thyroglobulin concentration were tested at different dilutions with standard 0.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Expected (ng/ml)</th>
<th>Measured (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 undiluted</td>
<td>-</td>
<td>179.50</td>
</tr>
<tr>
<td>1:2</td>
<td>89.75</td>
<td>84.75</td>
</tr>
<tr>
<td>1:4</td>
<td>44.88</td>
<td>47.57</td>
</tr>
<tr>
<td>1:8</td>
<td>22.44</td>
<td>28.46</td>
</tr>
<tr>
<td>S2 undiluted</td>
<td>-</td>
<td>25.69</td>
</tr>
<tr>
<td>1:2</td>
<td>12.85</td>
<td>15.05</td>
</tr>
<tr>
<td>1:4</td>
<td>6.42</td>
<td>6.97</td>
</tr>
<tr>
<td>1:8</td>
<td>3.21</td>
<td>3.83</td>
</tr>
</tbody>
</table>

10.5. Correlation
The NovaTec Thyroglobulin ELISA Kit was compared to another commercially available TG assay (TG Zentech Irma Kit). 66 serum samples were analysed according in both test system. The linear regression curve is:

TG Zentech = 1.029*TG NovaTec +2.069

R²=0.952
10.6. Recovery into the serum sample

In a sample, the presence of anti-Thyroglobulin self-antibodies influences Thyroglobulin assay and thus can cause inaccurate results. Therefore, it is necessary to carry out a clinical recovery test to confirm the accuracy of the result. This test should not be considered as a method for discovering anti-Thyroglobulin antibodies.

Procedure:
Dilute 1/2 of the serum sample with the Recovery solution, e.g. 50 µl of sample + 50 µl of Recovery Solution. The Thyroglobulin concentration in the Recovery Solution is 50 ng/ml.
Test the undiluted sample (S1) and the sample half diluted with the Recovery Solution (S2) according to the assay scheme.
The percentage of recovered Thyroglobulin for a sample is calculated as follows:

\[
\text{Recovery (\%)} = \frac{\text{ng/ml sample S2}}{(\text{ng/ml sample S1} + 50)/2} \times 100
\]

Recoveries less than 75 % or more than 120 % indicate interferences from anti-Thyroglobulin self-antibodies.

11. LIMITATIONS OF THE PROCEDURE

Bacterial contamination or repeated freeze-thaw cycles of the specimen may affect the absorbance values.
The results of the assay must be carefully interpreted and confirmed by clinical evaluations and further diagnostic tests.

12. 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!

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.

13. LITERATURE

14. ORDERING INFORMATION

Prod. No.: DNOV057  Thyroglobulin Determination (96 Determinations)
SCHEME OF THE ASSAY
Thyroglobulin

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>Standard 0 - 6</th>
<th>Control</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Standard 0 - 6</strong></td>
<td>-</td>
<td>50 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>-</td>
<td>-</td>
<td>50 µl</td>
<td>-</td>
</tr>
<tr>
<td><strong>Sample</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50 µl</td>
</tr>
<tr>
<td><strong>Biotin-Conjugate</strong></td>
<td>-</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 1.5 hours at 37 °C.**
Wash each well three times with 300 µl diluted Wash Solution.

<table>
<thead>
<tr>
<th></th>
<th>blank</th>
<th>Standard 0 - 6</th>
<th>Control</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HRP-Conjugate</strong></td>
<td>-</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 37 °C.**
Wash each well three times with 300 µl diluted Wash Solution.

<table>
<thead>
<tr>
<th></th>
<th>blank</th>
<th>Standard 0 - 6</th>
<th>Control</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TMB Subst.</strong></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).**

<table>
<thead>
<tr>
<th></th>
<th>blank</th>
<th>Standard 0 - 6</th>
<th>Control</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stop Solution</strong></td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

Photometric measurement at 450 nm

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DNOV057engl02092010-CR