








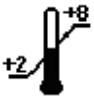






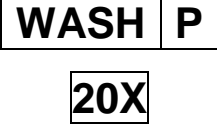













# **Free T4 ELISA**

**Enzyme Immunoassay for Quantitative Determination of  
Free Thyroxin in Human Serum**

*Instructions for use*

## 1. SYMBOL LEGEND

	In vitro diagnostic medical device		EC Declaration of conformity
	Catalogue number		Batch code
	Use by		Manufacturer
	Date of manufacture		Temperature limitation
	Caution, consult accompanying documents		Consult operating instructions
	Contains sufficient for <n> tests		Biological risks
	Coated microplate (96 wells)		Conjugate
	Wash solution, 20X concentrated		Substrate
	Calibrators		Stop solution
	Control		Optical density
	Reconstitute with specified volume of liquid		Deionized or distilled water
	Authorized Representative in the European Community		Trial
	Irritant		

**Warning**

## 2. INTENDED USE

**Free T<sub>4</sub> kit** is provided for the **quantitative** determination of **free thyroxin (free T<sub>4</sub>)** in human serum.

Thyroxin (T<sub>4</sub>) is one of the thyroid gland hormones with a molecular mass of 777 Da. Quantitative determination of serum free thyroxin is significant for evaluation of thyroid gland function.

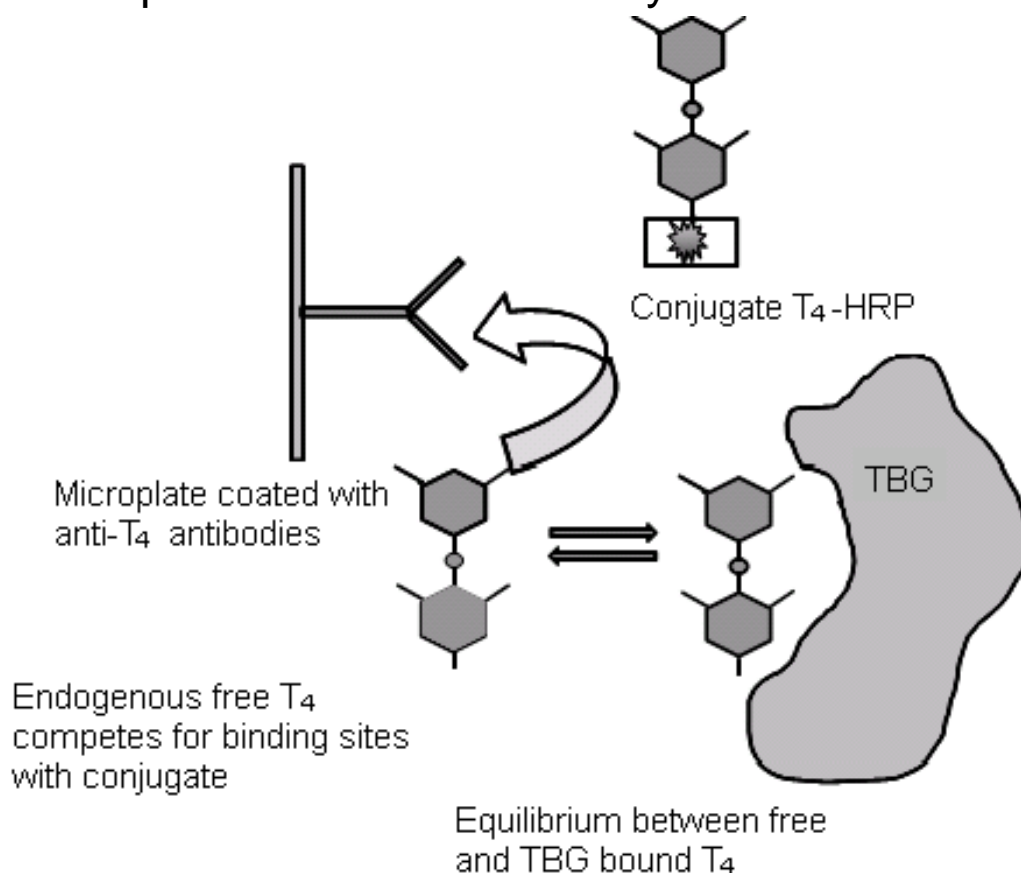
The bulk of serum T<sub>4</sub> persists in protein-bound form. The main T<sub>4</sub>-binding proteins are thyroxin-binding globulin (TBG), thyroxin-binding prealbumin and albumin. Concentration of non-bound form of T<sub>4</sub> (free T<sub>4</sub>) is very low, approximately 0.03% of the total concentration of circulating hormone. Namely free T<sub>4</sub> is considered to be responsible for biological activity of the hormone.

Free T<sub>4</sub> concentration does not depend on the concentration of binding proteins and remains normal when TBG levels are either increased (e.g. due to congenital pathology, pregnancy, intake of estrogens and oral contraceptives) or decreased (e.g. due to congenital pathology, intake of androgens and salicylates, renal insufficiency) and when the binding capacity of the proteins is diminished due to salicylates treatment. So, free T<sub>4</sub> level adequately reflects the actual thyroid status. Concentration of free T<sub>4</sub> is above normal in patients with thyroid hyperfunction or after intake of thyroxin preparations; it is below normal in the case of thyroid hypofunction.

### 3. PRINCIPLE OF THE TEST

**Free T<sub>4</sub> kit** is a competitive solid phase enzyme immunoassay. During the incubation free T<sub>4</sub> of tested samples and horseradish peroxidase (HRP) labeled T<sub>4</sub> bind to the antibodies coated onto the inner surface of the microplate wells until balance between them occurs. Separation of free and bound to antibodies T<sub>4</sub> and conjugate T<sub>4</sub>-peroxidase occurs while extracting the contents of the wells. The amount of bound conjugate is inversely proportional to the quantity of free T<sub>4</sub> in the sample (fig. 1).

During the incubation with TMB solution the colour is developing. The intensity of the colour is inversely proportional to the free T<sub>4</sub> concentration in specimens. The free T<sub>4</sub> concentration in the patient sample is read from a standard curve that is processed in each assay.



**Fig. 1. Assay scheme**

#### **4. STORAGE AND STABILITY OF THE KIT**

The expiration date of the kit is printed on the box label; the expiration date for each component is printed on the respective label.

**Free T4 kit** should be stored at 2-8°C upon receipt, preferably in the original kit box, until the expiration date.

Shelf life of the kit is 18 months.

After initial opening the kit is stable until the expiration date if stored at 2-8°C.

If used in several separate experiments, after initial opening kit contents should be stored as follows:

- unused strips: in a firmly closed resealable zipper bag at 2-8°C until the expiration date;
- vials with conjugate, calibrators and control (ready-to-use): at 2-8°C until the expiration date; vials with calibrators and control (reconstituted): at 2-8°C for no more than 1 month after opening;
- vial with substrate: at 2-8°C until the expiration date, protected from light;
- vials with concentrated Trial, concentrated wash solution and stop solution: at 2-8°C until the expiration date;
- wash solution and Trial solution prepared for use: at room temperature (18-25°C) for no more than 5 days, in a firmly closed bottle; wash solution prepared for use at 2-8 °C for no more than 4 weeks, in a firmly closed bottle.

#### **Damaged Test Kits**

In case of any severe damage of the test kit or components, it has to be informed in writing, during one week after

receiving the kit. Usage of severely damaged single components for a test run is not recommended.

## **5. SAMPLE COLLECTION AND STORAGE**

Collect blood by venipuncture in a tube without anticoagulants. Allow blood to clot. Centrifuge the specimens to separate serum from the blood corpuscles.

Do not use plasma, haemolyzed (bright red) or lipemic (milky) serum samples as well as samples containing sodium azide as preservative.

Store serum samples at 2-8°C for no more than 5 days. Aliquot and freeze samples for longer storage (-20°C and lower). Avoid repeated freezing.

## **6. EXPECTED VALUES**

Serum samples collected between 9 and 11 a.m. from 200 apparently healthy people, both males and females, between the ages of 18–45, were assayed with **Free T<sub>4</sub> kit**. Free T<sub>4</sub> concentration range in the samples was 10.0–23.2 pmol/L. These limits should be considered as guidelines only.

It is highly recommended each laboratory to determine its own reference range of free T<sub>4</sub> concentrations.

## **7. QUALITY CONTROL**

It is recommended to use control samples according to the state and federal regulations. The use of control samples is advised to assure the day to day validity of results.

## 8. REAGENT PREPARATION

Allow all the reagents to reach room temperature, and then thoroughly stir.

**MP** Keep **microplate** at room temperature (18-25°C) for at least 30 minutes before opening the bag. Place required number of strips onto strip holder. Place unused strips into the resealable zipper bag and reseal duly.

### **CAL CONTROL** Calibrators and Control

Liquid calibrators and control are ready to use.

Prepare lyophilized calibrators and control as follows. Gently tap on the vial caps to knock off all the dry matter. Open the vials and carefully place the caps upside down on the clean dry surface. Add 0.5 mL of distilled or deionized water to each vial with lyophilized calibrators and control, close vials with the corresponding caps and leave for 10 min at room temperature without stirring. Then stir gently avoiding foaming, until the dry matter is completely dissolved. Leave for another 10 minutes at room temperature stirring gently periodically. Make sure that no dry matter is left on the caps and walls of the vials.

**WASH P** Prepare required volume of **wash solution** by dilution of the concentrate 20-fold with distilled or deionized water. For example:

5 mL of **WASH P** **20X** + 95 mL of water.

Mix thoroughly, avoid foaming.

**SUB** Protect **substrate** from direct light.

## 9. SAMPLE PREPARATION

Allow samples to reach room temperature. Stir samples gently in order to ensure homogeneity.

## 10. PERFORMANCE CHARACTERISTICS OF THE ASSAY

### 10.1. Calibration-Traceability

**Free T4 kit** was calibrated via commercially available diagnostic kit, which had been standardized using equilibrium dialysis.

### 10.2. Specificity

Cross-reaction of anti-T<sub>4</sub> monoclonal antibodies with different thyroids is shown below.

Thyroid	Cross-reaction, %
L-Thyroxin	100.0
L-Triiodothyronine	8.0
L-Diiodotyrosine	0.001

### 10.3. Analytical Sensitivity

Analytical sensitivity of **Free T4 kit**, i.e. concentration, that can be distinguished from zero calibrator is 1.0 pmol/L. It was defined as mean OD of 10 replicates of calibrator 0 minus 2 SD.

### 10.4. Measurement Range

**Free T4 kit** was validated for measurement of free T<sub>4</sub> concentration within the concentration diapason of 1.0–100 pmol/L.

### 10.5. Measurement Units

In **Free T4 kit** the concentrations of calibrators are specified in pmol/L. To convert into ng/L, multiply the concentration in pmol/L by 0.777.

## 10.6. Intra- and Inter-Assay Variation (Precision)

For **intra-assay CV** determination, 8 serum samples were run, each in 9 replicates. The results are shown below.

Sample	Mean free T <sub>4</sub> concentration, pmol/L	Intra-assay CV	
		SD	CV, %
HS 1	11.1	0.22	2.0
HS 2	12.8	0.51	4.0
HS 3	12	0.46	3.8
HS 4	18.1	0.39	2.1
HS 5	18	0.19	1.0
HS 6	26.2	1.15	4.4
HS 7	50.2	3.71	7.4
HS 8	95.0	7.70	7.3

For **inter-assay CV** determination, 8 serum samples were assayed 3 times by different operators with 1-week interval. Each specimen was run in 9 replicates. The results are shown below.

Sample	Mean free T <sub>4</sub> concentration, pmol/L			Inter-assay CV	
	Assay 1	Assay 2	Assay 3	SD	CV, %
HS 1	11.1	11.1	10.2	0.52	4.8
HS 2	12.8	12.4	11.5	0.66	5.4
HS 3	12	12.5	11.2	0.66	5.5
HS 4	18.1	18.3	16.9	0.76	4.3
HS 5	18	17.8	17.1	0.48	2.7
HS 6	26.2	25.1	24.2	1.01	4
HS 7	50.2	50.2	44.8	3.10	6.4
HS 8	95	96.9	88.1	6.22	6.7

## 11. LIMITATION OF THE METHOD

Any clinical diagnosis should not be based on the results of in vitro diagnostic methods alone. For diagnosis establishment, a physician is supposed to consider all available clinical and laboratory findings.

## 12. SAFETY PRECAUTIONS

- **This kit is for in vitro diagnostic use only.** Operator should follow the manual closely in order to ensure reliable data. The manual is valid for the present kit only, within the listed composition. Any substitution of kit components is not allowed by CE regulations.

- Do not use kit or its components after the expiration date indicated on the label. Take into account stability period for reconstituted reagents.

- Do not mix or use together reagents from different lots of the kit except substrate, stop solution and wash solution.

- Do not use substrate, stop solution and wash solution supplied by other vendors.

- Use only “P”-labeled wash solution.

- Note that stop solution is 1N HCl solution. Avoid contacts with skin and mucosa. In case of contact rinse affected area thoroughly with plenty of water and seek medical advice.

- Take into account the following common procedural notes:

- always pipette reagents into wells immediately after washing procedure;

- avoid contamination of the solutions;

- in case of partial use of the kit, dispense only required volume of the reagent into the tray;
- do not pour unused reagents back into the original vials;
- avoid exposure to direct sunlight during incubations;
- always pipette reagents in the same order to minimize reaction time differences between wells; the total dispensing time for the calibrators, control and samples must not exceed 15 min;


- the incubation temperature for all the immunological reactions must be kept at 37°C;


- do not touch the bottom of the wells;

- calibrators should be measured in each separate assay.


It is also recommended to measure each time free T<sub>4</sub> concentration in the control.


- TMB solution should be colourless. Light colouring of solution is admissible. Avoid direct exposure of substrate to sunlight.

-  Source materials of human origin used for kit components preparation were tested and found negative for HBsAg, anti-HIV and anti-HCV antibodies. However, none of known laboratory test guarantees absence of these viral agents. Therefore, all kit components and patient's samples should be handled as potentially hazardous.

-  After usage strips, calibrators, control, specimens and all consumables which contacted with specimens during handling, storage or assay (tubes, vials, gloves, pipette tips etc.) should be collected separately and sterilized by autoclaving. Instead of autoclaving pipette tips may be sterilized by disinfectant treatment. After sterilization all components and


expendable materials may be utilized as non-dangerous garbage. Other components of the kit should be discarded into conventional garbage.

-  During manual washing procedure do not discard the contents of the wells directly to drainage. Use a container with disinfectant solution.

-  As the kit contains potentially hazardous material, the following precautions should be taken:

- do not smoke, eat or drink while performing the assay;
- always use protective gloves;
- never pipette material by mouth;
- in case of spilling, wipe up the spills promptly and wash affected area thoroughly using decontaminant.

- GLP including all general and individual regulations should be applied for the kit usage.



 As the kit contains irritant (**CONJ**, **CAL**, **CONTROL**), the following precautions should be observed:

- P261 - Avoid breathing spray;
- P272 - Contaminated work clothing should not be allowed out of the workplace;
- P280 - Wear protective gloves/protective clothing/eye protection;
- P302+P352 - IF ON SKIN: Wash with plenty of soap and water;
- P333+P313 - If skin irritation or rash occurs: Get medical advice/attention;
- P363 - Wash contaminated clothing before reuse;
- P501 - Dispose of contents/container in accordance with national regulation.

Precautionary statements according to Regulation EC № 1272/2008.

## 13. MATERIAL PROVIDED

### 13.1. Material Provided

<b>MP</b>	<b>Microplate:</b> 12 breakable 8-well strips (total 96 wells) coated with anti-T <sub>4</sub> monoclonal antibodies	1 pcs
<b>CONJ</b>	<b>Conjugate:</b> solution containing T <sub>4</sub> conjugated with HRP	18 mL, ready to use
<b>0-5 CAL</b>	<b>Free T<sub>4</sub> calibrators:</b> serum-based solutions or lyophilized preparations containing known free T <sub>4</sub> concentrations – 0; 3; 6; 12; 30; 100 pmol/L. The concentrations of calibrators may be different for schemes with or without shaking. For exact free T <sub>4</sub> concentrations, see vial labels. 	<b>6 vials,</b> 0.5 mL each; ready to use or lyophilized preparations
<b>CONTROL</b>	<b>Free T<sub>4</sub> control:</b> serum-based solution or lyophilized preparation containing known free T <sub>4</sub> concentration. The range of free T <sub>4</sub> concentration may be different for schemes with or without shaking. For exact range of free T <sub>4</sub> concentration see vial label. 	0.5 mL, ready to use or lyophilized preparation
<b>SUB</b>	<b>Substrate (TMB solution):</b> 3,3',5,5'-tetramethylbenzidine solution in citrate buffer containing hydrogen peroxide	14 mL, ready to use
<b>WASH P 20X</b>	<b>Wash solution P, 20X concentrated:</b> surfactant in buffered saline, sufficient for preparation of 560 mL of solution	2x14 mL, concentrated
<b>STOP</b>	<b>Stop solution:</b> 1N HCl solution	14 mL, ready to use

## **13.2. Equipment and Materials Required but not provided**

- 1-channel calibrated variable precision pipettes, with disposable tips;
- 8-channel calibrated variable precision pipette, with disposable tips;
- microplate incubator (37°C) or microplate incubator/shaker (37°C, shaking speed 500–800 rpm);
- manual or automatic equipment for rinsing wells;
- calibrated microplate reader (450 nm);
- vortex tube mixer;
- deionized or distilled water;
- graduated beaker and cylinder of appropriate volume;
- latex or plastic gloves;
- trays for pipetting reagents with 8-channel pipette;
- disinfectant;
- absorbent material (for manual wash).

## **13.3. Test Procedure**

**Free T4 kit** is designed for 96 tests. This is sufficient for the **quantitative assay** of 40 unknowns, 6 calibrators, 1 control and 1 blank (OD of TMB solution) in duplicates, provided that all the strips are used simultaneously.

### 13.3.1. Assay Procedure

#### 13.3.1.1. Protocol with shaking

(See assay scheme, section 13.5)

A. Pipette:

- 20  $\mu\text{L}$  of calibrators **CAL** (0-5), control **CONTROL** and patient's samples in duplicates into the respective wells;

**Leave wells A1-A2 empty for blank!**

B. Pipette:

- 150  $\mu\text{L}$  of conjugate **CONJ** into each well, except wells A1-A2.

**Note: total time of dispensing must not exceed 15 minutes, otherwise the test result may be unreliable, because the time of incubation will substantially vary for different samples.**

C. Incubate strips for **60 minutes while shaking (500–800 rpm) at 37°C.**

D. Wash 4 times, as described in section 13.3.2.

E. Pipette **100  $\mu\text{L}$  of substrate **SUB**** into each well (including blank); incubate **at room temperature in the dark for 15-30 minutes**, depending on the colour intensity, or **10 minutes while shaking (500-800 rpm) at 37°C.**

F. Pipette **100  $\mu\text{L}$  of stop solution **STOP**** into each well (including blank) in the same sequence and at the same speed as used for dispensing TMB substrate. Shake for 1–2 min at room temperature.

G. Read OD at **450 nm within 20 min.**

#### 13.3.1.2. Protocol without shaking

(See assay scheme, section 13.6)

A. Pipette:

- **20 µL** of **calibrators** **CAL** (0-5), **control** **CONTROL** and patient's samples in duplicates into the respective wells;

**Leave wells A1-A2 empty for blank!**

**B. Pipette:**

- **150 µL** of **conjugate** **CONJ** into each well, **except wells A1-A2.**

**Note: total time of dispensing must not exceed 15 minutes, otherwise the test result may be unreliable, because the time of incubation will substantially vary for different samples.**

**C. Incubate strips for 120 minutes at 37°C** (pre-shake for 1-2 minutes at room temperature).

**D. Wash 4 times, as described in section 13.3.2.**

**E. Pipette 100 µL of substrate** **SUB** into each well (including blank); incubate **at room temperature in the dark for 15-30 minutes**, depending on the colour intensity.

**F. Pipette 100 µL of stop solution** **STOP** into each well (including blank) in the same sequence and at the same speed as used for dispensing TMB substrate. Shake for 1–2 min at room temperature.

**G. Read OD at 450 nm within 20 min.**

### **13.3.2. Wash Procedure**

It is advisable to use an automatic microplate washer set at 4 wash cycles and a volume of 300 µL of wash solution per well per cycle.

If an automatic washer is not available, the wash procedure can be carried out manually as follows:

- remove the contents of the wells into a container with disinfectant;

- dispense 300  $\mu\text{L}$  of wash solution (prepared according to section 8) into each well, shake the plate carefully for 5–10 sec and remove the contents of the wells; repeat 4 times;
- strike the wells sharply on absorbent material to remove any liquid residue.

### **13.4. Data Processing**

If the reader cannot be adjusted to zero using the substrate blank in wells A1-A2, subtract mean OD value of wells A1-A2 from all OD values before further calculations.

*Example:*

OD (Cal 0) measured = 2.28 and OD (blank) = 0.06;

OD (Cal 0) calculated =  $2.28 - 0.06 = 2.22$

#### **13.4.1. Data Reliability (for OD Measured at 450 nm)**

The data should meet the following criteria:

- average blank OD (in wells A1-A2)  $\leq 0.100$ ;
- average OD of Cal 0  $\geq 1.0$  (after blank subtraction);
- control's concentration must fall within the acceptability range that is shown on the vial label.

If the data obtained do not meet the criteria, the results are considered unreliable and the test should be repeated.

### 13.4.2. Quantitative Determination

Specialized software for quantitative determination is recommended. Mean OD of calibrators are plotted versus their respective free  $T_4$  concentrations using 4PL or 5PL fit (see typical standard curve, fig. 2). Calculate concentration of free  $T_4$  in samples using standard curve.

Any extrapolation of the standard curve to free  $T_4$  concentration above the nominal value of the calibrator 5 (approximately 100 pmol/L) is forbidden.

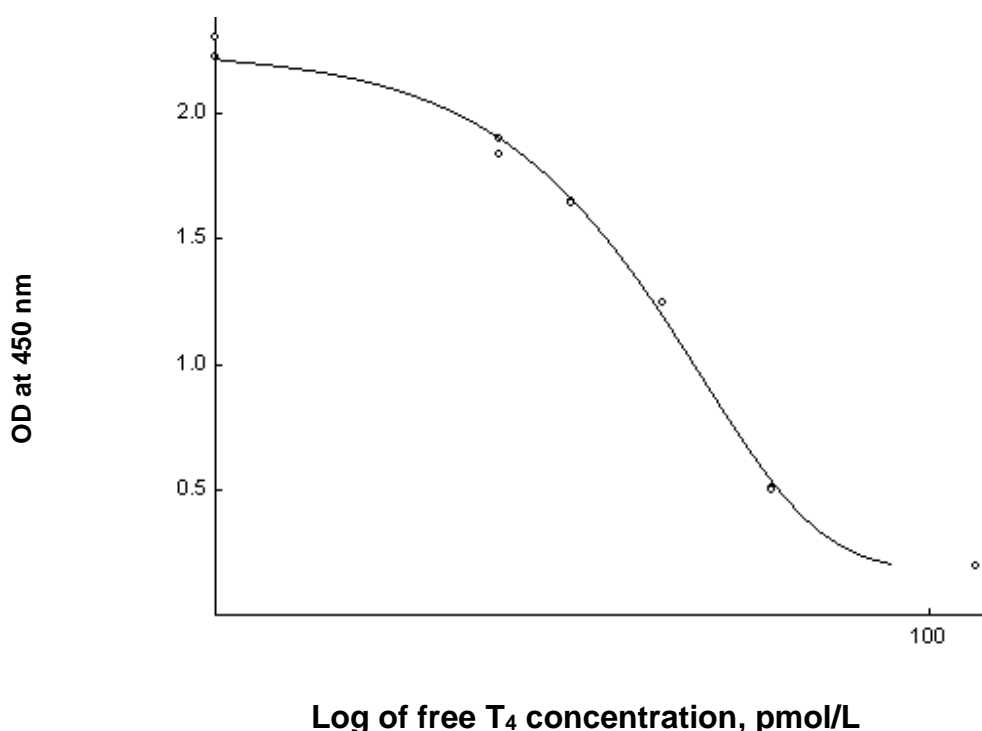


Fig. 2. Example of typical standard curve.  
**Do not use for evaluation of real assay data!**

### 13.5. Assay scheme with shaking

Reagents	Wells	«Blank»	<b>CAL</b>	Samples
			<b>CONTROL</b>	
<b>CAL</b>		–	20 µL	–
<b>CONTROL</b>				
Samples		–	–	20 µL
<b>CONJ</b>		–	150 µL	150 µL
Incubation No.1	60 min, 37°C, 500–800 rpm			
<b>WASH P</b> (diluted)	4 x 300 µL			
<b>SUB</b>	100 µL	100 µL	100 µL	100 µL
Incubation No.2	15-30 min, 18-25°C, in the dark			
	10 min, 37°C, 500–800 rpm			
<b>STOP</b>	100 µL	100 µL	100 µL	100 µL
Stirring	1–2 min, 18-25°C			
OD measuring	450 nm			
Calculations	Corresponding software			

### 13.6. Assay scheme without shaking

Reagents	Wells	«Blank»	<b>CAL</b>	Samples
			<b>CONTROL</b>	
<b>CAL</b>		–	20 µL	–
<b>CONTROL</b>				
Samples		–	–	20 µL
<b>CONJ</b>		–	150 µL	150 µL
Incubation No.1	120 min, 37°C (pre-shake for 1-2 minutes at room temperature).			
<b>WASH P</b> (diluted)	4 x 300 µL			
<b>SUB</b>	100 µL	100 µL	100 µL	100 µL
Incubation No.2	15-30 min, 18-25°C, in the dark			
<b>STOP</b>	100 µL	100 µL	100 µL	100 µL
Stirring	1–2 min, 18-25°C			
OD measuring	450 nm			
Calculations	Corresponding software			

#### **BIOGENIX INC. PVT. LTD.**

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