AB Diagnostic Systems

REF **DK.061.01.8** Σ **96** IVD **For *In vitro* Diagnostic Use**

INSTRUCTIONS FOR USE
abia Rubella IgG
Enzyme immunoassay for the qualitative and quantitative determination of IgG antibodies to *Rubella Virus* in human serum or plasma

This Package Insert provides information for Professional Use of the kit.
The kit contains sufficient reagents for 96 (one breakable plate) assays including controls; the kit is intended for manual testing with a possibility of fractional (one strip) use of the kit or for use of the kit on open type automated analyzer for enzyme immunoassay.

I. INTENDED USE

The abia Rubella IgG kit is intended for the detection of IgG antibodies specific to *Rubella virus* in human serum (plasma) by a microplate enzyme immunoassay.

This kit is for diagnostic use by a trained laboratory professional and will not be sold to the general public. All the reagents are for professional *in vitro* diagnostic use only.

The results of this or any other diagnostic assay should be used and interpreted only in the context of the overall clinical picture.

II. INTRODUCTION

Rubella is a viral exanthematous infectious disease caused by *Rubella virus*, a single-stranded RNA virus belonging to the Togavirus group. The illness follows a typically benign clinical course with rare complications and is subclinical in a large proportion of cases. Symptomatology is generally mild, characterized by fever, malaise, a maculopapular rash of three to five days' duration and, possibly, coryza and conjunctivitis. The disease is usually accompanied by lymphadenopathy. Infection confers lifelong immunity. Infection from *Rubella virus* is particularly disastrous if contracted during the first four months of gestation. If not immunologically protected, women infected during pregnancy run a high risk of embryofoetal damage. Congenital rubella causes a wide range of severe defects, many of which are permanent and adversely affect later development (cataract, deafness, hepatosplenomegaly, psychomotor retardation, bone alterations, cardiopathies, neuropathies). The first humoral immune response to infection is the synthesis of specific anti-*Rubella virus* IgM antibody which reaches high serum levels two weeks after the rash and lasts in the circulation for one to two month(s). Specific IgG antibody generally appears a few days after the onset of rash, about one week after IgM develops. It rapidly increases to reach a plateau six to ten weeks after the onset of symptoms and then progressively decreases to a level (15-200 IU/ml) lasting for the whole life. Reinfection, completely asymptomatic, is accompanied by moderately increased levels of specific IgG. Correct detection of IgM and IgG antibodies to *Rubella virus* provides an essential tool for diagnosing and following up acute infection, for assessment of immune status in fertile women, and therefore for adopting suitable prophylaxis in susceptible women of child-bearing age. Since when a vaccine was made available, the assay of IgG to *Rubella virus* has been widely used to determine seroconversion of the recipient after vaccination.

III. PRINCIPLE OF THE TEST

The abia Rubella IgG plates are coated with *Rubella virus* antigen. Serum to be tested is diluted and incubated with the precoated plate. In this step anti-Rubella specific antibodies are bound to the immobilized *Rubella virus* antigen. Non specific antibodies are removed by washing. Anti-human IgG conjugated to horseradish peroxidase (HRP) is added and incubated. In this step the HRP-conjugate is bound to the prebound antigen-antibody complex. The unbound components are removed by washing. After addition of the solution containing TMB and hydrogen peroxide, the wells with bound enzyme develop a blue colour which is converted to yellow after the reaction has been stopped with sulphuric acid. The colour intensity is directly proportional to the concentration of specific antibodies in the specimen and can be read at 450/620-680 or 450 nm.

IV. CONTENT OF THE KIT abia Rubella IgG

4.1 Contents of the reagent kit.

Table 1

LABEL	NATURE OF THE REAGENTS	PRESENTATION
Rubella virus-Ag Coated Strips	Polystyrene stripped 96-well plate (breakable wells) coated with *Rubella virus* antigen. Store at 2-8 °C until expiration date.	1 plate
Conjugate	Antibodies to human IgG, conjugated with HRP enzyme with addition of 1M Tris HCl buffer (pH 7.4), Tween®20 (0.11%) and bovine serum albumin (3.85%). Preserving agents: 0.10% ProClin 300, 0.004% gentamicin sulfate, 0.09% phenol. Transparent or slightly opalescent yellow colored liquid. Store at 2-8 °C until expiration date in a tightly sealed vial.	1 vial 11.0 ml
Calibrator 1, Inactivated	Control sample, containing 10 IU/ml Anti-Rubella IgG*. Preserving agents: 0.10% phenol, 0.10% sodium azide. Transparent or slightly opalescent red colored liquid. Store at 2-8 °C until expiration date in a tightly sealed vial.	1 vial 1.2 ml
Calibrator 2, Inactivated	Control sample, containing 40 IU/ml Anti-Rubella IgG*. Preserving agents: 0.10% phenol, 0.10% sodium azide. Transparent or slightly opalescent red colored liquid. Store at 2-8 °C until expiration date in a tightly sealed vial.	1 vial 1.2 ml

Calibrator 3, Inactivated	Control sample, containing 80 IU/ml Anti-Rubella IgG*. Preserving agents: 0.10% phenol, 0.10% sodium azide. Transparent or slightly opalescent red colored liquid. Store at 2-8 °C until expiration date in a tightly sealed vial.	1 vial 1.2 ml
Calibrator 4, Inactivated	Control sample, containing 160 IU/ml Anti-Rubella IgG*. Preserving agents: 0.10% phenol, 0.10% sodium azide. Transparent or slightly opalescent red colored liquid. Store at 2-8 °C until expiration date in a tightly sealed vial.	1 vial 1.2 ml
Negative Control, Inactivated	Control sample, not containing species-specific antibodies to *Rubella virus*. Preserving agents: 0.10% phenol, 0.10% sodium azide. Transparent or slightly opalescent green colored liquid. Store at 2-8 °C until expiration date in a tightly sealed vial.	1 vial 2.5 ml
Preliminary Sample Diluent	Sample buffer that is used for preliminary dilution of samples. Preserving agent: 0.09% sodium azide. Transparent or slightly opalescent violet-blue colored liquid. Store at 2-8 °C until expiration date in a tightly sealed vial.	1 vial 11.0 ml
Sample Diluent	Sample buffer that is used to dilute samples before analysis. Preserving agent: 0.006% thimerosal, 0.10% sodium azide. Transparent or slightly opalescent pink colored liquid. Store at 2-8 °C until expiration date in a tightly sealed vial.	1 vial 11.0 ml
Washing Solution (concentrated 25-fold)	Phosphate-saline solution (pH 7.4-7.7). Transparent or slightly opalescent colorless or light yellow liquid, sediment may form that dissolves completely at 35-39 °C and shaking. Store at 2-8 °C until expiration date in a tightly sealed vial.	1 vial 50.0 ml
Stopping Reagent	Sulfuric acid solution (H_2SO_4) 0.2M. Transparent colorless liquid. Store at 2-8 °C until expiration date in a tightly sealed vial.	1 vial 25.0 ml

TMB-Substrate	Tetramethylbenzidine in citric acid buffer, containing H_2O_2. Transparent colorless liquid. Store at 2-8 °C until expiration date in a tightly sealed vial.	1 vial 14.0 ml
Plate for preliminary dilution of sera	Polystyrene plate with transparent wells.	1 plate
Protective films for EIA plates		2
Polyethylene bag with a Zip-Lock		1
Disposable plastic dishes for liquid reagents		2
Disposable tips		16

*The Calibrators were calibrated using a WHO International Standard Anti Rubella Immunoglobulin, Human NIBSC code: RUBI-1-94.

V. PRECAUTIONS

The reliability of the results depends on correct implementation of the following requirements:

- The temperature in the lab should be 18-24 °C.
- Inspect the contents of the box: check the vials and labels integrity. If labels are lost or labels/vials damage, vials should be disposed, and **kit cannot be used**.
- Do not use expired reagents.
- Do not mix reagents from different lots within a given test run.
- Do not carry out the test in the presence of reactive vapors (acid, alkaline, aldehyde vapors) or dust that could alter the enzyme activity of the conjugates.
- Use glassware thoroughly washed and rinsed with deionized water or preferably, disposable material.
- Do not allow the microplate to dry between the end of the washing operation and the reagent distribution.
- The enzyme reaction is very sensitive to metal ions. Consequently, do not allow any metal element to come into contact with the various conjugate or substrate solutions.
- Use a new distribution tip for each sample.
- Well washing is a critical step in this procedure: respect the recommended number of washing cycles and make sure that all wells are completely filled and then completely emptied. Incorrect washing may lead to inaccurate results.
- Never use the same container to distribute conjugate and color development solution.

- Check the pipettes and other equipment for accuracy and correct operation.
- Do not change the assay's procedure.
- Use high quality water.
- Avoid exposure of the reagents to excessive heat or sunlight during storage and incubation.

VI. HEALTH AND SAFETY INSTRUCTIONS

- All reagents included in the kit are intended for "*in vitro* diagnostic use".
- Human origin material used in the preparation of Calibrators 1-4 and Negative Control has been tested and found negative for HBsAg, antibodies to hepatitis C virus and antibodies to human immunodeficiency virus (HIV-1 and HIV-2), antigen p24 HIV-1.
- Because no known test method can offer complete assurance that infectious agents are absent, handle reagents and patients samples as if capable of transmitting infectious disease.
- Do not eat, drink, smoke, or apply cosmetics where immunodiagnostic materials are being handled.
- Any equipment directly in contact with specimens and reagents as well as washing solutions should be considered as contaminated products and treated as such.
- Wear lab coats and disposable gloves when handling reagents and samples and thoroughly wash your hands after handling them.
- Avoid spilling samples or solutions containing samples. Wipe spills immediately and decontaminate affected surfaces.
- Avoid any contact of the TMB-Substrate and the Stopping Reagent with the skin and mucosa.
- Provide adequate ventilation.
- All materials contacted with specimens or reagents, including liquid and solid wastes, should be inactivated by validated procedures (autoclaving or chemical treatment) and disposed in accordance with applicable local law regulations.

Warning!

Conjugate contains ProClin 300.
H317: May cause an allergic skin reaction.
P261: Avoid breathing vapours.
P280: Wear protective gloves/protective clothing/eye protection/face protection.
P302 + P352: IF ON SKIN: Wash with plenty of water.
P333 + P313 If skin irritation or rash occurs: Get medical advice/ attention.

Danger!

Calibrator 1, Calibrator 2, Calibrator 3, Calibrator 4, Negative Control, Sample Diluent contain sodium azide.
H312: Harmful in contact with skin.
P280: Wear protective gloves/protective clothing/eye protection/face protection.
P302 + P352 IF ON SKIN: Wash with plenty of soap and water.
P312: Call a POISON CENTER or doctor/physician if you feel unwell.

Danger!

Stopping Reagent contains 0.2 M/L sulfuric acid.
H314 Causes severe skin burns and eye damage.
P280 Wear protective gloves/protective clothing/ eye protection/face protection.
P303 + P361 + P353 IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.
P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P310 Immediately call a POISON CENTER or doctor/ physician.

VII. MATERIALS AND EQUIPMENT REQUIRED BUT NOT PROVIDED WITH THE KIT:

- Distilled or deionized water.
- Automatic or semiautomatic, adjustable or preset single-channel and multi-channel pipettes with a changeable volume for a set of liquids.
- Disposable pipette tips.
- Microplate incubator at (37.0 ± 1.0) °C.
- Automatic microplate washer.
- Microplate reader equipped with 450 nm or with 450 and 620-680 nm filters.
- Laboratory clock.
- Open type automated analyzer with 450 nm or with 450 and 620-680 nm filters (for automated procedure).

VIII. COLLECTION AND HANDLING OF SPECIMENS

Collection of blood samples should be implemented according to the current practices. Serum, plasma (citrate, heparin, EDTA) may be used. Separate serum or plasma from blood cells as soon as possible to avoid any haemolysis. Extensive haemolysis may affect test performance. Specimens with observable particulate matter should be clarified by centrifugation prior to testing. Suspended fibrin particles or aggregates may yield falsely positive results. Do not heat the samples.

Samples can be stored at 2-8 °C not more than for 48 hours; they may be deep-frozen at -20 °C. Samples that have been frozen and defrosted more than 1 time cannot be used. Samples with expressed bacterial growing, hemolysis, hyperlipidemia must not be analyzed.

IX. PREPARATION OF THE REAGENTS

1. Ready to use reagents:

- **Rubella virus-Ag Coated Strips.** Strips are wrapped in a sealed foil-lined bag. Open the bag and remove the tray plate. Select the number of Coated Strips required for the assay. Return unused strips in the bag. After the bag has been opened the Coated Strips are stable during the shelf life of the kit at 2-8 °C, provided that the foil-lined bag is resealed in Zip-Locked plastic bag. The silica gel bag should not be removed from the foil packaging.
- **Conjugate;**
- **Calibrators 1-4;**
- **Negative Control;**
- **Preliminary Sample Diluent;**

- **Sample Diluent;**
- **Stopping Reagent;**
- **TMB-Substrate.**

2. Reagents to prepare:

- **Working Washing Solution**. Thoroughly shake Washing Solution concentrate. To make Working Washing Solution take required amount of concentrate and mix with distilled or deionized water (1:24 ratio) in a separate vial. Thoroughly mix the solution. The prepared Working Washing Solution is stable for 14 days at 18-24 °C or for 28 days at 2-8 °C.

The required volumes of Working Washing Solution for the certain number of strips or plate are tabulated in Table 2.

Table 2

Number of strips to be used		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**1 well**
Working Washing Solution	Washing Solution (×25), ml	3.0	6.0	9.0	12.0	15.0	18.0	21.0	24.0	27.0	30.0	33.0	40.0	0.2
	High quality water, ml	72.0	144.0	216.0	288.0	360.0	432.0	504.0	576.0	648.0	720.0	792.0	960.0	4.8

3. Storage of unused reagents

After opening the vials the unused components of the kit: Calibrators 1-4, Negative Control, Preliminary Sample Diluent, Sample Diluent, Washing Solution (concentrated 25-fold), Stopping Reagent can be stored in tightly sealed vials until the kit expiration date at 2-8 °C. Coated Strips can be stored until the kit expiration date at 2-8 °C.

Conjugate, TMB-Substrate can be stored in tightly sealed vials within 3 months at 2-8 °C.

X. TEST PROCEDURE

Note: Before use, allow reagents to reach room temperature (18-24 °C) for 30 min.

Step	The assay procedure
1	Add 90 µl of Preliminary Sample Diluent into the wells of the plate for preliminary samples dilution and 10 µl of the tested serum (plasma) samples. Carefully mix by pipetting. Violet-blue color should change to blue-green.
2	**For qualitative detection** Add 100 µl of control samples into the wells: Calibrator 1 – to 2 wells, Calibrator 4 – to 2 wells, Negative Control – to 2 wells. **For quantitative detection** Add 100 µl of control samples into the wells: Calibrator 1 – to 2 wells, Calibrator 2 – to 2 wells, Calibrator 3 – to 2 wells, Calibrator 4 – to 2 wells, Negative Control – to 2 wells.

3	Add 90 µl of Sample Diluent and 10 µl of the preliminary diluted samples to the rest of the wells (the final serum dilution ratio is 1:100). Carefully mix fluid in wells by gentle pipetting. Cover the strips with a protective film.
4	Incubate for **30 min** in a microplate incubator at (37.0 ± 1.0) °C.
5	Aspirate the contents of the wells and wash the plate **4 times** with the Working Washing Solution. Add into each well not less than 380 µl of Working Washing Solution and remove Washing Solution into the container with disinfecting solution. Do not leave any fluid in the wells. Use of an automatic microplate washer is strongly recommended. Incomplete washing will adversely affect the assay precision.
6	Add 100 µl of Conjugate to all the wells of the plate. Cover the plate with a protective film.
7	Incubate for **30 min** in a microplate incubator at (37.0 ± 1.0) °C.
8	Remove fluid from wells, wash the plate **4 times** as described in step 5.
9	Add 100 µl of TMB-Substrate into all the wells.
10	Incubate at 18-24 °C for a **20 min** in a dark place.
11	Add 150 µl of Stopping Reagent into wells to stop the reaction results are read by microplate plate reader at wavelength of 450 nm, with reference filter at 620-680 nm. Reading of the absorbance at 450 nm only is possible.

Scheme of the assay is represented in Annex.

Automated analyzer

Validated test protocols and dilution tables of reagent working solutions for different EIA-analyzers can be obtained from the manufacturer upon request (see section XIV). For the instrumentation without established validated protocol follow the section “TEST PROCEDURE” and ensure all requirements described in the section “PRECAUTIONS” are fulfilled. All protocols for automated analyzers must be fully validated before use.

When preparing working reagent solutions for automated EIA procedure, it is necessary to consider “dead” volume of vials and containers used for loading working solutions in the EIA analyzer.

XI. RESULTS

Test Validation

For the test to be valid the following criteria must be met. If these criteria are not met the test should be considered invalid and should be repeated.

Negative Control: The absorbance value should not be more than 0.200.

Calibrator 1: The absorbance value should not be less than 0.200.

Calibrator 4: The absorbance value should not be less than 1.000.

Qualitative results

The presence or absence of antibodies against *Rubella virus* is determined by the ratio of the OD of each sample to the calculated Cut-Off value.

Calculate the Cut-Off:

Cut-Off = average OD value of Calibrator 1

Interpretation of Results:

Sample is positive, if the OD value is ≥ Cut-Off.
Sample is negative, if the OD value is < Cut-Off.

If sample remains equivocal after repeat testing, repeat analysis of this sample in pair with serum taken 10-15 days later to determine the increase of anti-Rubella-IgG concentration.

Quantitative results

The concentration of anti-Rubella-IgG calculate only for positive samples.

Calculate average OD values of each Calibrator. Draw the graph representing dependence of optical density from concentration of anti-Rubella-IgG in calibration samples:

X-axis: optical density (OD) of Calibrators;
Y-axis: AB concentration (CAB), IU/ml.

If sample OD is over the average meaning of Calibrator 4 retest it, the analysis should be repeated with the sample preliminary diluted 1:40 (the final serum dilution ratio is 1:400). Calculating the concentration of Anti-Rubella-IgG take into account the dilution factor (1:4).

The concentration level of anti-Rubella-IgG over 10 IU/ml is recommended as protective.

XII. PERFORMANCE CHARACTERISTICS

1. Analytical sensitivity

The analytical sensitivity was calculated by adding 2 standard deviations from the mean of 20 replicate analyses of Negative Control and was found to be 0.74 IU/ml. Therefore, the analytical sensitivity of the abia Rubella IgG kit does not exceed 1 IU/ml.

2. Interferences

The specimens, containing up to 0.3 mg/ml of bilirubin, up to 6 mg/ml of hemoglobin and up to 20 mg/ml of lipids, do not affect the performance quality of the assay and may be studied with the reliable result.

3. Cross reactivity

No cross reactivity was found for samples with anti-HSV 1,2.

4. Evaluation of diagnostic sensitivity of abia Rubella IgG has been performed with using 108 serum samples. Diagnostic sensitivity is 100% (95% Cl: 93.8-100%).

5. Diagnostic specificity

Evaluation of diagnostic specificity of abia Rubella IgG has been performed with using 108 serum samples. Diagnostic specificity is 100% (95%Cl: 92.9-100%).

6. Trueness. Agreement with certified reference measurement procedure

The abia Rubella IgG was compared with the "Rubella Virus IgG" (Virion\Serion, Germany) and "Enzygnost Anti-Rubella Virus/IgG" (SIEMENS, USA). 117 serum and plasma samples are tested.

abia Rubella IgG	"Rubella Virus IgG" (Virion\Serion, Germany)			"Enzygnost Anti-Rubella Virus/IgG" (SIEMENS, USA)		
	Positive	Negative	Total	Positive	Negative	Total
Positive	60	2	62	59	0	59
Negative	0	52	52	2	53	55
Total	60	54	114	61	53	114
Percent Positive Agreement	100% (95Cl: 94.0-100.0%)			96.8% (95Cl: 89.0-99.1%)		
Percent Negative Agreement	96.3% (95Cl: 87.5-99.0%)			100% (95Cl: 93.2-100%)		
Overall Percent Agreement	98.2% (95Cl: 93.8-99.5%)			98.3% (95Cl: 93.9-99.5%)		

7. Precision

The precision of the abia Rubella IgG was determined by 20 days × 3 samples × 2 replicates covering the measuring range.

Repeatability of the abia Rubella IgG

Data	Sample Pool #1	Sample Pool #2	Sample Pool #3
Mean (ng/ml)	34.64	12.48	5.45
S_r	2.74	0.58	0.32
CV (%) < 8%	7.9	4.7	5.9

Between-run precision of the abia Rubella IgG

Data	Sample Pool #1	Sample Pool #2	Sample Pool #3
Mean (ng/ml)	34.64	12.48	5.45
S_{rr}	2.61	0.68	0.41
CV (%) < 8%	7.5	5.5	7.5

8. Recovery

Samples were prepared by adding defined amount of anti-Rubella-G (Calibrator 1) to Control Serum.

Sample	Measured concentration, IU/ml	Expected concentration, IU/ml	**Recovery, %**
Serum sample	21.53	-	-
Calibrator 1	10.00	-	-
Serum sample + Calibrator 1	15.28	15.76	96.91%

9. Linearity

The linearity of the test was investigated using serial dilutions of patient's serum samples with different concentrations anti-Rubella IgG. The abia Rubella IgG is linear in the measurement range 12.39 to 139.10 IU/ml.

10. Hook effect

The hook effect was not observed up to concentration of anti-Rubella IgG 500 IU/ml.

XIII. LIMITS OF THE TEST

- No single serological test should be used for a final diagnosis. All clinical and laboratory data should be taken into account.
- Very limited number of studies has been conducted in a group of neonates. Serological diagnosis of these patients is more difficult. This is due to the fact that maternal IgG passes transplacentally to the fetus and can affect the outcome of the testing in this group.

XIV. CONDITIONS OF STORAGE AND TRANSPORTATION

Expiry date is indicated on the packaging.

Keep in dark dry place at 2-8 °C.

Transportation should be done at 2-8 °C. Transportation at 9-25 °C is allowed for not more than ten (10) days. Freezing is prohibited.

AB Diagnostic Systems GmbH
Sportfliegerstraße 4, Berlin, 12487, Germany
Tel. +49 30 208987160, Fax: +49 30 208987199
E-Mail: info@ab-ds.de, www.ab-ds.de

XV. REFERENCES

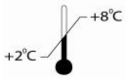
1. Rubella (German Measles). Katz S.L. In Zinssmer Microbiology. 18th Edition. Jolik, Willett, Amos 8ed Chapt. 75:1067 1985.

2. Rubella virus. Herman K.L. In Manual of Clinical Microbiology. 3rd Edition. Lennette, Balows, Hausler, Truant, (ed). Chapt. 86:862 1980.

3. Voller A. and Bidwell D.E. (1975) A Simple Method For Detecting Antibodies To Rubella. Br. J. Exp. Pathol. 56:338.

4. Gravell M., Dorcett, P.H. Gutenson, O and Ley, A.C. (1977). Detection of Antibody to Rubella virus by Enzyme-Linked Immuno-Sorbent Assay. J. Infect. Dis. 136:S300.

XVI. EXPLANATION OF SYMBOLS

IVD	For in vitro diagnostic use		
	Manufacturer		Consult Instruction for use
	Date of manufacture CCYY-MM		Symbol “exclamation mark”
REF	Catalog number		Symbol “corrosion”
	Sufficient for		Symbol “health hazard”
LOT	Batch code	**Danger!** **Warning!**	Signal words
	Storage temperature limitation		Keep away from sunlight
	Expiry date CCYY-MM-DD		Keep dry
	Top		Fragile, handle with care

Annex

Scheme of the assay

1	**Add**	90 µl of Preliminary Sample Diluent and 10 µl of the samples (conduct on the plate for preliminary dilution of samples)
2	**Add**	100 µl of Calibrator 1, Calibrator 2, Calibrator 3, Calibrator 4, Negative Control
3	**Add**	90 µl of Sample Diluent
4	**Add**	10 µl of preliminary diluted samples
5	**Incubate**	30 min, (37.0 ± 1.0) °C, microplate incubator
6	**Wash the plate**	Working Washing Solution, not less than 380 µl, 4 times
7	**Add**	100 µl of Conjugate
8	**Incubate**	30 min, (37.0 ± 1.0) °C, microplate incubator
9	**Wash the plate**	Working Washing Solution, not less than 380 µl, 4 times
10	**Add**	100 µl of TMB-Substrate
11	**Incubate**	20 min, 18-24 °C in a dark place
12	**Add**	150 µl of Stopping Reagent
13	**Read the optical density**	450 nm/620-680 nm or 450 nm