BIOGENIX
INC. PVT. LTD.

CA 125 ELISA

Enzyme Immunoassay for Quantitative Determination of Cancer Antigen 125 in Human Serum

Instructions for use

1. SYMBOL LEGEND

IVD	In vitro diagnostic medical device	CE	EC Declaration of conformity
REF	Catalogue number	LOT	Batch code
	Use by		Manufacturer
	Date of manufacture		Consult operating instructions
	Temperature limitation		Biological risks
EC REP	Authorized Representative in the European Community	CONJ	Conjugate
MP	Coated microplate (96 wells)	SUB	Substrate
WASH P 20X	Wash solution, 20X concentrated	STOP	Stop solution
CAL	Calibrators	DIL	Sample diluent
CONTROL	Control	OD	Optical density
	Contains sufficient for <n> tests	H2O	Deionized or distilled water
TRIAL 5000X	Trial, 5000X concentrated	RCNS	Reconstitute with specified volume of liquid

2. INTENDED USE

CA 125 kit is provided for the **quantitative determination of cancer antigen 125 (CA 125) in human serum**.

CA 125 is a mucoglycoprotein with a molecular weight of over 200 kDa. Normally in adults CA 125 exists in two forms: free and membrane-bound. Membrane-bound antigen can be identified on the surface of epithelial cells of fallopian tubes, cervix, endometrium, bronchi, mammary gland and sudoriferous gland. High concentrations of free form can be detected in seminal liquid, breast milk, vaginal secretions, saliva, bronchoalveolar and intraperitoneal liquids. In bloodstream CA 125 persists in low concentrations. Increased concentration of serum CA 125 is a sign of ovarian pathology (either benign or malignant).

Quantitative determination of serum CA 125 is used for monitoring of patients with ovarian cancer for estimation of treatment efficiency, early identification of recurrences and asymptomatic dissemination of residual tumor. Diagnostic value of method depends on histological type of tumor: it is the highest in the case of serous ovarian carcinomas in comparison with other types of carcinoma (e.g. mucinous carcinoma).

3. PRINCIPLE OF TEST

OncoEIA-CA 125 kit is a “sandwich” type of solid-phase enzyme immunoassay, based on two monoclonal antibodies that are specific for different epitopes of CA 125 molecule. One of these antibodies is conjugated with horseradish peroxidase (HRP); the other is conjugated with biotin. CA 125 molecules from the serum sample bound to both antibodies and are immobilized onto the inner surface of microwells coated with streptavidin. Then the wells are washed with wash solution to remove any material not bound to the inner surface of the wells. Quantity of bound conjugate is directly proportional to CA 125 level in tested sample.

During the incubation with TMB solution the colour is developing. The intensity of the colour is directly proportional to CA 125 concentration in specimens. CA 125 concentration in the patient sample is read from a standard curve that is processed in each assay.

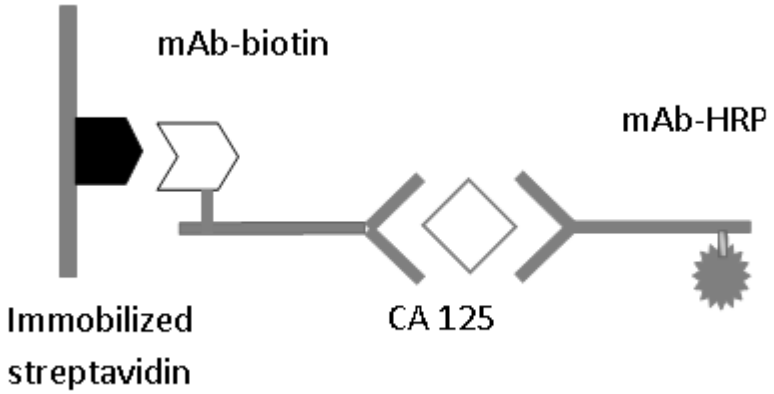

Fig. 1. Assay scheme

4. STORAGE AND STABILITY OF THE KIT

Expiry date of the kit is printed on the box label; expiry date for each component is printed on the respective label.

OncoEIA-CA 125 kit should be stored at +2…+8 °C upon receipt, preferably in the original kit box, until the expiration date. Storage at +25 °C is allowed but for no more than 15 days.

Shelf life of the kit is 12 months. After initial opening the kit is stable for 12 months if stored at +2…+8 °C.

If used in several separate experiments, after initial opening kit contents should be stored as follows but never used longer than the expiration date:

- unused strips: in a firmly closed resealable zipper bag, concentrated wash solution and stop solution at +2…+8 °C until the expiration date;
- vials with conjugate, calibrators and control (ready-to-use), sample diluents: at +2…+8 °C for no more than 12 months after opening;
- vials with calibrators and control (reconstituted): at +2…+8 °C for no more than 3 months after opening;
- vial with substrate: at +2…+8 °C until the expiration date, protected from light;
- wash solution prepared for use: at room temperature (+18…+25 °C) for no more than 5 days or at +2…+8°C for no more than 4 weeks, in a firmly closed bottle;
- Trial solution prepared for use: at room temperature (+18…+25 °C) for no more than 5 days, in a firmly closed bottle;

- vials with concentrated Trial: at +2…+8 °C until the expiration date.

Damaged Test Kits

In case of any severe damage of the test kit or components, Alkor Bio 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. After clotting, the serum is separated by centrifugation.

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

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

6. EXPECTED VALUES

The range of CA 125 concentration up to 35 U/mL was classified as normal. CA 125 concentration is above normal in 50% of women with primary ovarian cancer and in 80 % of women with metastatic ovarian cancer. These limits should be considered as guidelines only.

It is highly recommended each laboratory determine its own reference range of CA 125 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 PREPERATION

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:

14 mL of **WASH P** **20X** + 266 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.

If the expected CA 125 concentration in the sample is higher than concentration of **calibrator 5**, the sample should be diluted consistently 20-fold and 400-fold with **sample diluent** **DIL** as follows:

Sample 1 (20-fold dilution): 190 µl of sample diluent **DIL** + 10 µl of serum sample, mix thoroughly.

Sample 2 (400-fold dilution): 190 µl of sample diluent **DIL** + 10 µl of Sample 1, mix thoroughly.

If the expected CA 125 concentration in the Sample 2 is higher than in **calibrator 5**, the sample should be diluted with **sample diluent** **DIL** as follows:

Sample 3 (800-fold dilution): 100 µl of sample diluent **DIL** + 100 µl of Sample 2, mix thoroughly.

10. PERFORMANCE CHARACTERISTICS OF THE ASSAY

10.1. Calibration-Traceability

OncoEIA-CA 125 kit was calibrated via internal manufacturer standard.

10.2. Specificity

According to Product specification, provided by the supplier no cross-reaction was detected between anti-CA 125 monoclonal antibodies used in the assay and CEA, CA 19-9, CA 15-3 and CA 72-4 cancer antigens.

No cross-reaction between anti-CA 125 monoclonal antibodies used in the assay and CEA, CA 19-9, CA 15-3 and CA 72-4 cancer antigens was detected. However, have been reported that in case of increased biotin uptake by patients, biotin interference with immunoassays might take place which may lead to false results.

10.3. Analytical Sensitivity

Analytical sensitivity of **OncoEIA-CA 125 kit** i.e. concentration that can be distinguished from zero calibrator is 1,6 U/mL. It was defined as mean OD of 10 replicates of calibrator 0 plus two standard deviations.

10.4. Measurement Range

OncoEIA-CA 125 manual and Alisei kits were validated for measurement of CA 125 concentration within the concentration diapason (without dilution) of 1,6 – 1000 U/mL.

10.5. Hook Effect

For **OncoEIA-CA 125 kit high dose hook effect** was not detected for concentrations up to 24 000 U/mL.

10.6. Precision (intra- and inter-assay CV)

Intra-assay CV

To determine intra-assay CV 8 serum samples were assayed in 9 replicates each. The results are shown below.

Sample	Mean CA 125 concentration, U/mL	Intra-assay CV	
		SD	CV, %
HS 1	17.1	1.7	7.6
HS 2	46.9	3.9	8.1
HS 3	93.9	7.5	6.4
HS 4	245.6	16.8	7.4
HS 5	349.6	18.7	5.7
HS 6	431.3	25.9	6.7
HS 7	832.5	56.5	8.0
HS 8	880.0	48.1	6.6

Inter-assay CV

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

Sample	Mean CA 125 concentration, U/mL			Inter-assay precision	
	1 assay	2 assay	3 assay	SD	CV, %
HS 1	29.9	32.447	31.65	1.26	4.0
HS 2	59.4	54.512	58.727	2.66	4.6
HS 3	114.1	100.01	104.38	7.19	6.8
HS 4	369.6	323.9	346.1	22.87	6.6
HS 5	444.7	392.51	413.77	26.23	6.3
HS 6	506.2	531.7	533.77	15.33	2.9
HS 7	718.4	687.1	714.4	17.0	2.4
HS 8	923.7	935.0	895.2	20.5	2.2

10.7. Dilution Parallelism of Serum Samples

Serial dilutions of three human serum samples with predetermined CA 125 concentration in sample diluent were assayed with **OncoEIA-CA 125 kit** with the following result:

Sample	Dilution	Measured concentration, U/mL	Expected concentration, U/mL	Measured /expected concentration ratio, %
1	Undiluted	703.4		
	1:2	321.0	351.8	109.6
	1:4	170.3	175.8	103.3
	1:8	87.3	87.9	100.7
	1:16	48.6	44.0	90.4
2	Undiluted	329.9		
	1:2	153.1	165.0	93.0
	1:4	234.5	82.5	105.0
	1:8	82.5	41.2	96.0
	Undiluted	306.2		
3	1:2	157.5	153.13	103.0
	1:4	82.7	76.56	108.0
	1:8	41.1	38.28	107.0

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 expiry 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 Alkor Bio wash solution.
- Note that stop solution is 1 N 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 the wells;
  - -do not touch the bottom of the wells;
  - calibrators should be measured in each separate assay. It is also recommended to measure each time CA 125 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, DIL, 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

MP	**Microplate:** 12 breakable 8-well strips (total 96 wells) coated with streptavidin	1 pcs
CONJ	**Conjugate:** solution contains anti-CA 125 monoclonal antibodies conjugated with HRP and anti-CA 125 monoclonal antibodies conjugated with biotin	18 mL, ready to use
0–5 CAL	**CA 125 calibrators:** protein-based solution or lyophilized preparations containing known CA 125 concentrations – 0, 25,100, 250, 500, 1000 U/mL (approximate values). The concentrations of calibrators may be different for schemes with or without shaking.For exact CA 125 concentrations, see vial labels	6 vials, 0.5 mL each; ready to use or lyophilized preparations
CONTROL	**CA 125 control:** protein-based solution or lyophilized preparation containing known CA 125 concentration. The range of CA 125 concentration may be different for schemes with or without shaking. For exact range of CA 125 concentration see vial label	0.5 mL, ready to use or lyophilized preparation
SUB	**Substrate (TMB solution):** 3,3',5,5'-tetramethylbenzidine solution in citrate buffer containing hydrogen peroxide	14 mL, ready to use
WASH P 20X	**Wash solution P, 20X concentrated:** surfactant in buffered saline, sufficient for preparation of 560 mL of solution	2x14 mL, concentrated
STOP	**Stop solution:** 1 N HCl solution	14 mL, ready to use
DIL	**Sample diluent**	3.0 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 (+35 -+39 °C) or incubator/shaker (+35 - +39 °C, shaking speed 500–750 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

CA 125 kit is designed for 96 tests. This is sufficient for 40 unknowns, 6 calibrators, 1 control and 1 blank (OD of TMB solution) in duplicates, provided that all the strips are used simultaneously.

13.1.1. Assay Procedure

13.3.1.1.Protocol with shaking
(See assay scheme, section 13.5)
All samples should be tested in duplicates.

A. Pipette**:**

- **50 µL** of calibrators **CAL**, control **CONTROL** and patient's samples in duplicates, into the respective wells **except wells A1-A2 (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 **for 60 minutes at +37 °C while shaking (500–750 rpm)**

D. Wash 5 times, as described in section 13.3.2.

E. Pipette **100 µL** of **substrate** **SUB** into each well (including blank); incubate strips **at room temperature** (+18…+25 C) **in the dark for 15-30 minutes**, depending on the colour intensity, or **10 minutes while shaking (500-750 rpm) at +37 C**.

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 (+18…+25 °C).

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

13.3.1.2. Protocol without shaking

(See assay scheme, section 13.6.)

All samples should be tested in duplicates.

A. Pipette**:**

- **50 μL** of calibrators **CAL**, control **CONTROL** and patient's samples in duplicates, into the respective wells **except wells A1-A2 (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 **for 2 hours at (+18…25^{0}C) without shaking (pre-shake the microplate for 1-2 minutes at (+18…25^{0}C).**

D. Wash 5 times, as described in section 13.3.2.

E. Pipette **100 μL** of **substrate SUB** into each well (including blank); incubate strips **at room temperature** (+18…+25 C) **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 (+18…+25 °C).

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

13.3.2. Wash Procedure

It is advisable to use an automatic microplate washer set at 5 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 μL 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 5 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 5) measured = 2.28 and OD (blank) = 0.06;

OD (Cal 5) 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) ≤0.09;
- -average OD of Cal 5 ≥ 1.5 (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 values of the calibrators at 450 nm versus their respective CA 125 concentrations using 4PL or 5PL fit (see typical standard curve, fig. 2). The arithmetical mean OD of the two «Blank» wells is used for zero setting of device.

Any extrapolation of the standard curve to CA 125 concentration above the nominal value of calibrator 5 is forbidden. In this case the sample should be additionally diluted accordingly to 9. Multiply the measured concentration of pre-diluted samples by corresponding dilution factor.

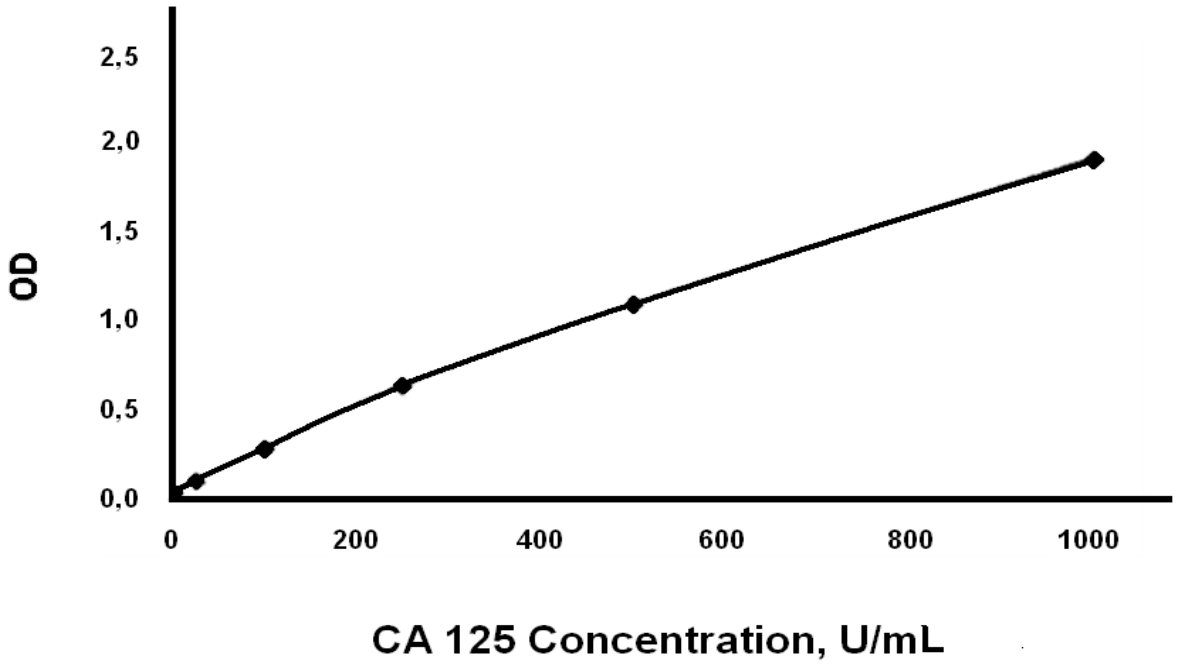

Fig. 2. Example of typical standard curve.
Do not use for evaluation of real assay data!

13.5. Assay scheme with shaking

Wells / Reagents	«Blank»	**CAL** **CONTROL**	Samples
CAL **CONTROL**	–	50 µL	–
Samples	–	–	50 µL
CONJ	–	150 µL	150 µL
Incubation No.1	60 minutes at +37 °C, 500–750 rpm		
WASH P (diluted)	5 x 300 µL		
SUB	100 µL	100 µL	100 µL
Incubation No.2	15–30 min, +18…+25 °C, in the dark or		
	10 min, +37°C, 500–750 rpm		
STOP	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

Wells / Reagents	«Blank»	**CAL** **CONTROL**	Samples
CAL **CONTROL**	–	50 µL	–
Samples	–	–	50 µL
CONJ	–	150 µL	150 µL
Incubation No.1	120 minutes,+18…25^{0}C (pre-shake for 1-2 minutes at room temperature)		
WASH P (diluted)	5 x 300 µL		
SUB	100 µL	100 µL	100 µL
Incubation No.2	15–30 min, +18…+25 °C, in the dark		
STOP	100 µL	100 µL	100 µL
Stirring	1–2 min, +18…+25 °C		
OD measuring	450 nm		
Calculations	Corresponding software		

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