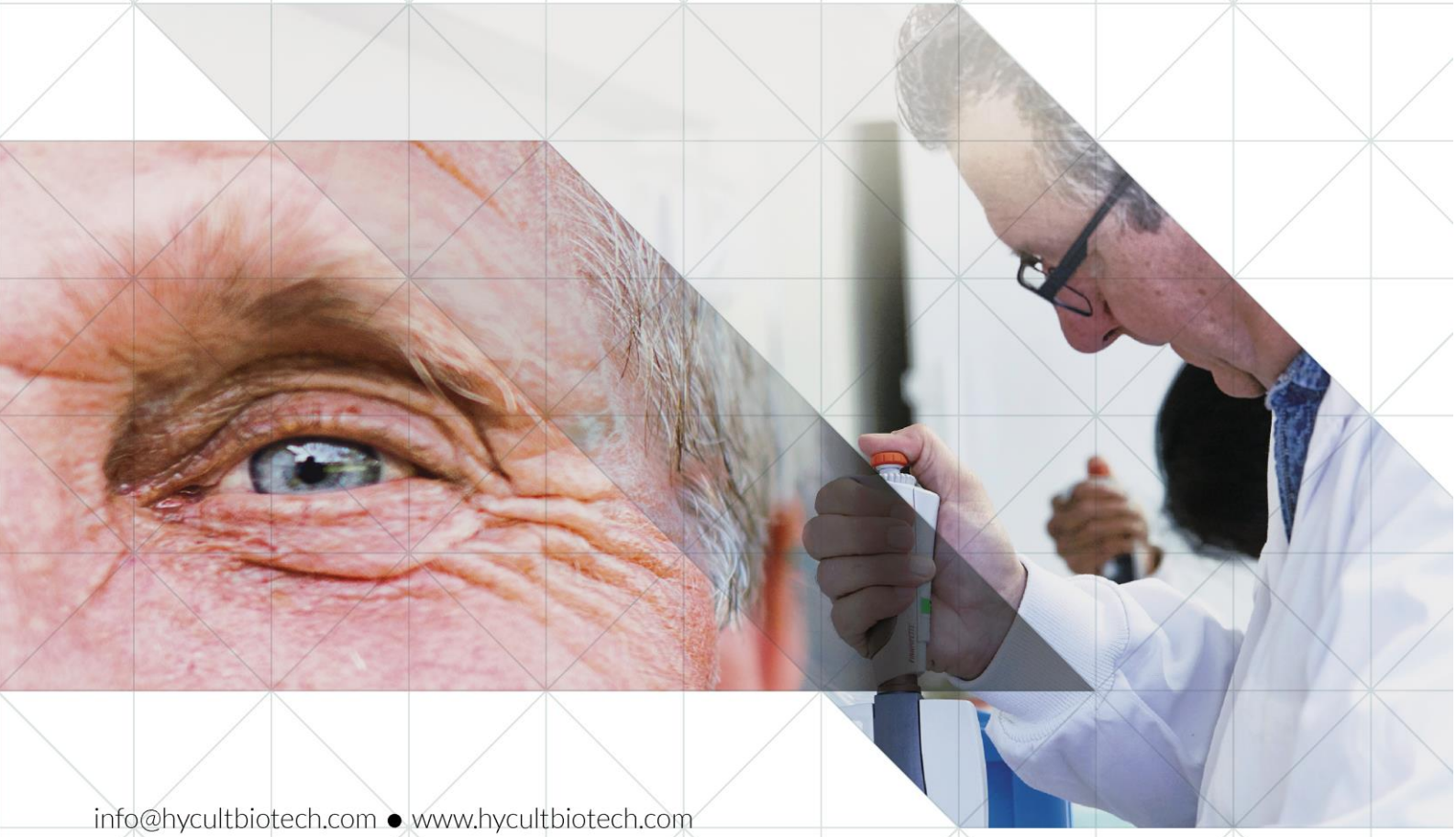
Competitive Nitrotyrosine ELISA

HIT501

Edition 10-18

ELISA KIT
PRODUCT INFORMATION & MANUAL

Read carefully prior to starting procedures!
For use in laboratory research only
Not for clinical or diagnostic use



info@hycultbiotech.com ● www.hycultbiotech.com

Note that this user protocol is not lot-specific and is representative for the current specifications of this product. Please consult the vial label and the Certificate of Analysis for information on specific lots. Also note that shipping conditions may differ from storage conditions.

For research use only. Not for use in or on humans or animals or for diagnostics. It is the responsibility of the user to comply with all local/state and federal rules in the use of this product. Hycult Biotech is not responsible for any patent infringements that might result from the use or derivation of this product.

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1. INTENDED USE

The competitive Nitrotyrosine ELISA kit is to be used for *in vitro* quantitative determination of nitrotyrosine in serum, plasma and faeces samples. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic procedures.

The analysis should be performed by trained laboratory professionals.

2. INTRODUCTION

Nitrotyrosine (NO) has been identified as a marker of inflammation and NO production. Nitrotyrosine is formed in presence of the active metabolite NO. Nitrosylation of the amino acid tyrosine occurs both for free tyrosine and protein bound tyrosine. Various pathways including the formation of peroxinitrite lead to nitrotyrosine production. Since nitrotyrosine is a stable end product of peroxynitrite oxidation, assessment of its plasma concentration may be useful as a marker of NO-dependent damage *in vivo*. Since nitrogen oxide species (NOX) is only an indicator for enhanced NO production, protein associated nitrotyrosine might be a more suitable marker for damage induced by reactive nitrogen intermediates derived from NO. Furthermore, most proteins have a longer half life in the circulation than NOX levels. The presence of nitrotyrosine has been detected in various inflammatory processes including atherosclerotic plaques, celiac disease, rheumatoid arthritis, chronic renal failure and septic shock. In normal serum and plasma nitrotyrosine levels are low or sometimes even undetectable.

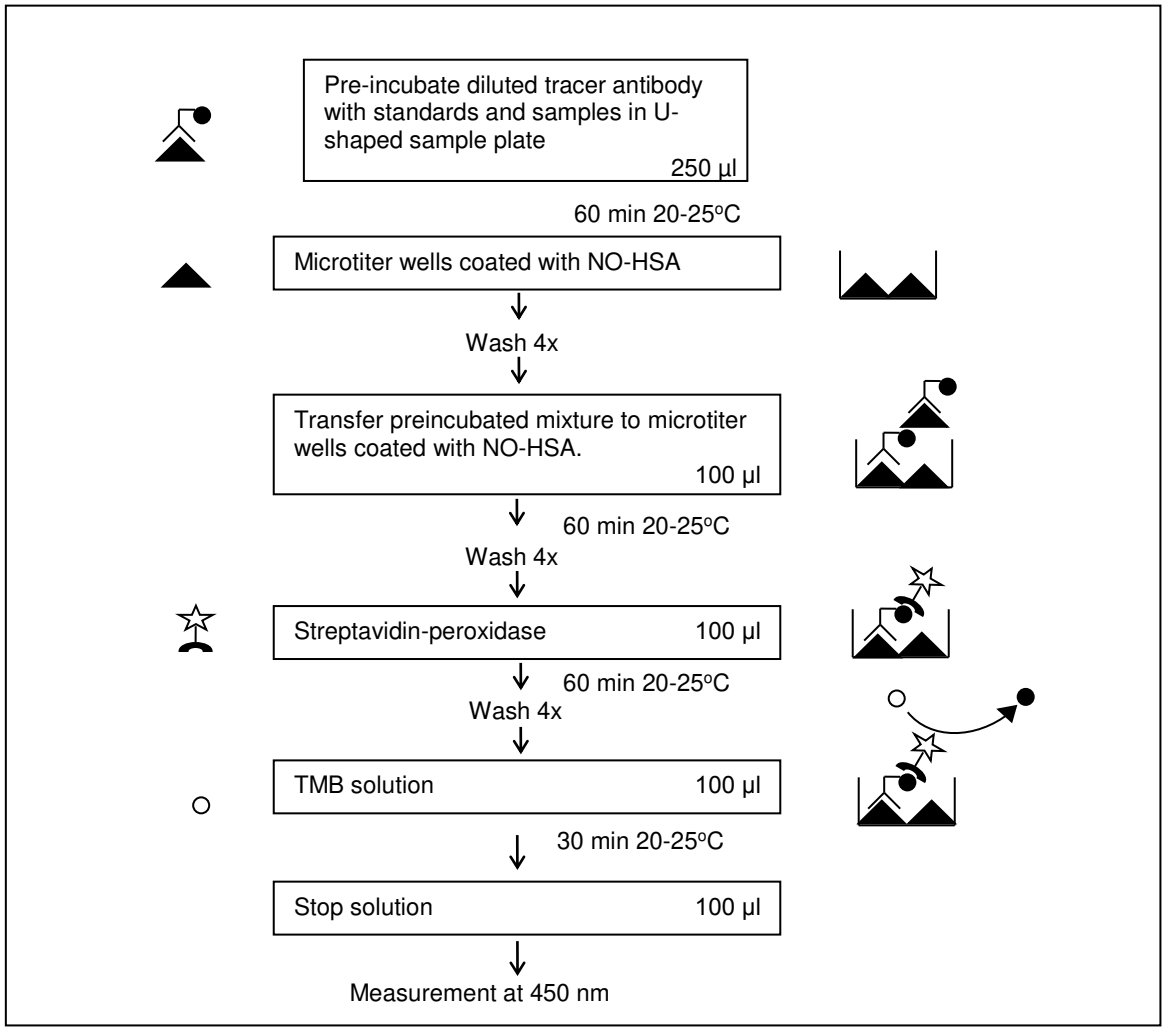
3. KIT FEATURES

- Working time of 3½ hours.
- Minimum concentration which can be measured is 46.9 nM.
- Measurable concentration range of 46.9 to 3000 nM.
- Working volume of 100 µl/well.

Cross-reactivity

The ELISA detects nitrotyrosine containing proteins. Since the assay detects a modified amino acid the assay is useful for proteins of all species.

4. PROTOCOL OVERVIEW



- The competitive Nitrotyrosine ELISA is a ready-to-use solid-phase enzyme-linked immunosorbent assay based on the inhibition principle with a working time of 3½ hours.
- The efficient format of a plate with twelve disposable 8-well strips allows free choice of batch size for the assay.
- Samples and standards are pre-incubated with biotinylated tracer antibody in U-shape microtiter plate.
- Pre-incubated samples and standards with the biotinylated tracer antibodies are incubated on Nitrotyrosine-HSA (NO-HSA) coated strips.
- Streptavidin-peroxidase conjugate will bind to the biotinylated tracer antibody.
- Streptavidin-peroxidase will react with the substrate, tetramethylbenzidine (TMB).
- The enzyme reaction is stopped by the addition of oxalic acid.
- The absorbance at 450 nm is measured with a spectrophotometer. A standard curve is obtained by plotting the absorbance (linear) versus the corresponding concentrations of the known standards of the NO-HSA standards (log).
- The nitrotyrosine concentration of samples, which are run concurrently with the standards, can be determined from the standard curve.

5. KIT COMPONENTS AND STORAGE INSTRUCTIONS

Kit component	Cat.#	Quantity	Color code
Wash buffer 20x	WB21	1 vial (60 ml)	Colorless
Dilution buffer 10x	DB81	1 vial (15 ml)	Green
Standard		2 vials, lyophilized	White
Tracer, biotinylated 100x		2 vials, 0.25 ml lyophilized	Blue
Streptavidin peroxidase 100x	CON03	1 tube, 0.25 ml in solution	Brown
TMB substrate	TMB050	1 vial (11 ml)	Brown
Stop solution	STOP110	1 vial (22 ml)	Red
U-shaped microtiter plate	UPL01	1 plate	
12 Microtiter strips, pre-coated		1 plate	
Certificate of Analysis		1	
Manual		1	
Data collection sheet		2	

Table 1

- Upon receipt, store individual components at 2 - 8°C. Do not freeze.
- Do not use components beyond the expiration date printed on the kit label.
- The standard and conjugate in lyophilized form and the streptavidin-peroxidase in concentrated solution are stable until the expiration date indicated on the kit label, if stored at 2 - 8°C.
- The exact amount of standard is indicated on the label of the vial and the Certificate of Analysis.
- The standard is single use. After reconstitution the standard cannot be stored for repeated use.
- The tracer is single use. After reconstitution the conjugate cannot be stored for repeated use.
- The streptavidin-peroxidase can only be stored in concentrated solution and is not stable when stored diluted.
- Upon receipt, foil pouch around the plates should be vacuum-sealed and unpunctured. Any irregularities to aforementioned conditions may influence plate performance in the assay.
- Return unused strips immediately to the foil pouch containing the desiccant pack and reseal along the entire edge of the zip-seal. Quality guaranteed for 1 month if stored at 2 - 8°C.

Materials required but not provided

- Calibrated micropipettes and disposable tips.
- Distilled or de-ionized water.
- Plate washer: automatic or manual.
- Polypropylene tubes.
- Calibrated ELISA plate reader capable of measuring absorbance at 450 nm.
- Adhesive covers can be ordered separately. Please contact your local distributor.
- Centrifuge for 1 ml tubes.

6. WARNINGS AND PRECAUTIONS

- For research use only, not for diagnostic or therapeutic use.
- This kit should only be used by qualified laboratory staff.
- Do not add under any circumstances sodium azide as preservative to any of the components.
- Do not use kit components beyond the expiration date.
- Do not mix reagents from different kits and lots. The reagents have been standardized as a unit for a given lot. Use only the reagents supplied by manufacturer.
- The assay has been optimized for the indicated range. Do not change the range.
- Open vials carefully: vials are under vacuum.
- It is advised to spin down streptavidin-peroxidase tubes before use.
- Do not ingest any of the kit components.
- Kit reagents contain 2-chloroacetamide as a preservative. 2-Chloroacetamide is harmful in contact with skin and toxic if swallowed. In case of accident or if you feel unwell, seek medical advise immediately.
- The TMB substrate is light sensitive, keep away from bright light. The solution should be colorless until use.
- The stop solution contains 2% oxalic acid and can cause irritation or burns to respiratory system, skin and eyes. Direct contact with skin and eyes should be strictly avoided. If contact occurs, rinse immediately with plenty of water and seek medical advise.
- Incubation times, incubation temperature and pipetting volumes other than those specified may give erroneous results.
- Do not reuse microwells or pour reagents back into their bottles once dispensed.
- Handle all biological samples as potentially hazardous and capable of transmitting diseases.
- Use polypropylene tubes for preparation of standard and samples. Do not use polystyrene tubes or sample plates.

7. SAMPLE PREPARATION

Collection and handling

Serum or plasma

Collect blood using normal aseptic techniques. Blood samples should be kept on ice. If serum is used, separate serum from blood after clotting at room temperature within 1 hour by centrifugation (1500xg at 4°C for 15 min). Transfer the serum to a fresh polypropylene tube.

If plasma is used, separate plasma from blood within 20 minutes after blood sampling by centrifugation (1500xg at 4°C for 15 min). Transfer the plasma to a fresh polypropylene tube.

Faeces

Nitrotyrosine can be measured in faeces if samples are extracted, for example using the following extraction buffer: 0.1 M Tris, 0.15 M NaCl, 1.0 M urea, 10 mM CaCl2, 5 g/l BSA and 0.25 mM thimerosal (pH 8.0). Add 5 ml extraction buffer to 100 mg sample (giving a dilution factor of 51, assuming the density of faeces to be 1 g/ml). Vortex samples and filter the samples to remove coarse particles (> 0.6 mm). Shake the filtrate for 20 minutes and centrifuge samples: 10,000xg at 4°C for 20 minutes. Use supernatant for analysis.

Storage

Store samples below -20°C, preferably at -70°C in polypropylene tubes. Storage at -20°C can affect recovery of inhibitory capacity. Use samples within 24 hours after thawing. Avoid multiple freeze-thaw cycles which may cause loss of endoblock LBP activity and give erroneous results.

Before performing the assay, samples should be brought to room temperature (18 – 25°C) and mixed gently. Prepare all samples (controls and test samples) prior to starting the assay procedure. Avoid foaming.

Dilution procedures

Serum or plasma

Samples can be measured accurately if diluted at least 10x with supplied dilution buffer in polypropylene tubes.

Faeces

Samples can be measured accurately if diluted at least 8x with supplied dilution buffer in polypropylene tubes.

Remark regarding recommended sample dilution

The mentioned dilution for samples is a minimum dilution and should be used as a guideline. The recovery of inhibitory compound from an undiluted sample is not 100% and may vary from sample to sample. When testing less diluted samples it is advisable to run recovery experiments to determine the influence of the matrix on the detection of inhibitory compound.

Do not use polystyrene tubes or sample plates for preparation or dilution of the samples.

Guideline for dilution of samples

Please see table 2 for recommended for sample dilutions. Volumes are based on a total volume of at least 230 µl of diluted sample, which is sufficient for one sample in duplicate in the ELISA. For dilution of samples we recommend to use at least 10 µl of sample.

	Dilution	Pre-dilution	Amount of sample or pre-dilution required	Amount of Dilution buffer required
1.	10x	Not necessary	25 µl (sample)	225 µl
2.	20x	Not necessary	15 µl (sample)	285 µl
3.	50x	Not necessary	10 µl (sample)	490 µl
4.	100x	Not necessary	10 µl (sample)	990 µl
5.	500x	Recommended: 10x (see nr.1)	10 µl (pre-dilution)	490 µl
6.	1000x	Recommended: 10x (see nr.1)	10 µl (pre-dilution)	990 µl
7.	2000x	Recommended: 20x (see nr.2)	10 µl (pre-dilution)	990 µl
8.	5000x	Recommended: 50x (see nr.3)	10 µl (pre-dilution)	990 µl

Table 2

8. REAGENT PREPARATION

Allow all the reagents to equilibrate to room temperature (20 – 25°C) prior to use. Return to proper storage conditions immediately after use.

Wash buffer

Prepare wash buffer by mixing 60 ml of 20x wash buffer with 1140 ml of distilled or de-ionized water, which is sufficient for 1 x 96 tests. In case less volume is required, prepare the desired volume of wash buffer by diluting 1 part of the 20x wash buffer with 19 parts of distilled or de-ionized water.

Dilution buffer

Prepare dilution buffer by mixing 15 ml of the 10x dilution buffer with 135 ml of distilled or de-ionized water, which is sufficient for 1 x 96 tests. In case less volume is required, prepare the desired volume of dilution buffer by diluting 1 part of the 10x dilution buffer with 9 parts of distilled or de-ionized water. Concentrated dilution buffer may contain crystals. In case the crystals do not disappear at room temperature within 1 hour, concentrated dilution buffer can be warmed up to 37°C. Do not shake the solution.

Standard

The standard is reconstituted by pipetting the amount of dilution buffer mentioned on the CoA in the standard vial. Transfer 250 µl of the reconstituted standard to well A1 of the U-shaped microtiter plate. Prepare a serial dilution of the reconstituted standard with dilution buffer in the U-shaped microtiter plate as shown in Figure 1*.

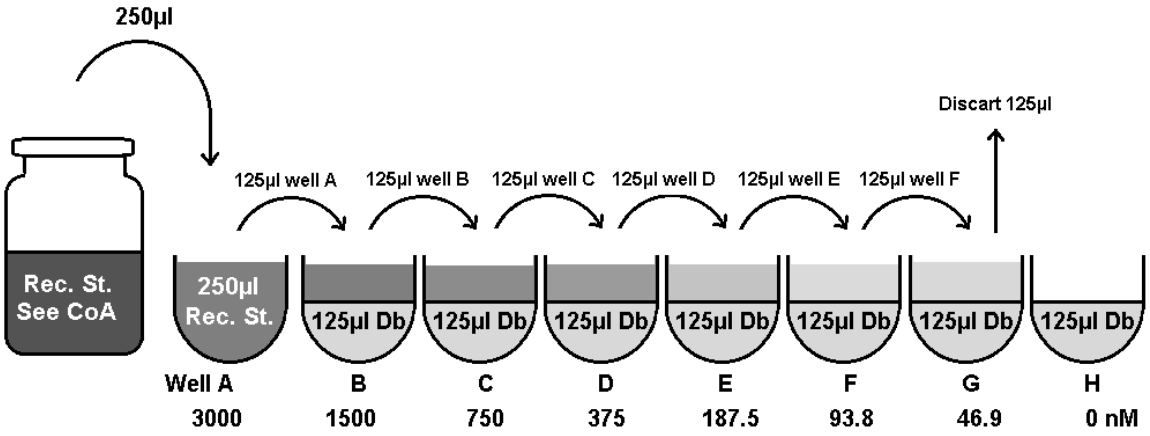


Figure 1

*) CoA: Certificate of Analysis, Rec. St: Reconstituted Standard, Db: Dilution buffer.

Tracer

The tracer is reconstituted by pipetting 0.25 ml distilled or de-ionized endotoxin free water. After reconstitution the tracer cannot be stored for repeated use. Dilute the reconstituted 0.25 ml tracer with 24.75 ml dilution buffer, which is sufficient for 1 x 96 tests. In case less volume is required, prepare the desired volume of tracer by diluting 1 part of the reconstituted tracer with 99 parts of dilution buffer.

Streptavidin-peroxidase solution

It is advised to spin down streptavidin-peroxidase tubes before use. Prepare the streptavidin-peroxidase solution by mixing 0.25 ml of the 100x streptavidin-peroxidase solution with 24.75 ml dilution buffer, which is sufficient for 2 x 96 tests. In case less volume is required, prepare the desired volume of streptavidin-peroxidase solution by diluting 1 part of the 100x streptavidin-peroxidase solution with 99 parts of dilution buffer.

9. ELISA PROTOCOL

Bring all reagents to room temperature (20 - 25°C) before use.

1. Determine the number of test wells required and fill out the data collection sheet. Return the unused strips of the NO-HSA coated microtiter plate to the storage bag with desiccant, seal and store at 2 - 8°C.
   Please notice the amount of standards and samples with tracer (total of 250µl) in the U-shape plate is sufficient for a duplicate determination of standards, samples and controls. Therefore, double the amount of wells for the coated microtiter plate are needed.
2. Prepare the standard dilution series in the U-shaped plate. Prepare the samples and add 125 µl to the U-shaped plate.
3. Add 125 µl of the diluted tracer to the 125 µl of standards and samples in the U-shaped plate. Gently mix by pipetting.
4. Cover the tray and tap the tray to eliminate any air bubbles. Be careful not to splash liquid onto the cover.
5. Incubate U-shaped plate for 1 hour at room temperature.
6. Wash the microtiter plate coated with NO-HSA 4 times with wash buffer using a plate washer or as follows*:
   a. Add 200 µl of wash buffer to each well, wait 20 seconds, empty the plate by inverting plate and shaking contents out over the sink, keep inverted and tap dry on a thick layer of tissues.
   b. Repeat the washing procedure 6a three times.
   c. Empty the plate and intensively tap on a thick layer of tissues. Tap the plate as dry as possible.
7. Transfer 100 µl in duplicate of standard, samples, or controls mixed with tracer from the U-shaped plate into appropriate wells in the coated microtiter plate. Do not touch the side or bottom of the wells.
8. Cover the tray and tap the tray to eliminate any air bubbles. Be careful not to splash liquid onto the cover.
9. Incubate for 1 hour at room temperature.
10. Repeat the wash procedure described in step 6.
11. Add 100 µl of diluted streptavidin-peroxidase to each well, using the same pipetting order as applied in step 7. Do not touch the side or bottom of the wells.
12. Cover the tray and incubate the tray for 1 hour at room temperature.
13. Repeat the wash procedure described in step 6.
14. Add 100 µl of TMB substrate to each well, using the same pipetting order as applied in step 7. Do not touch the side or bottom of the wells.
15. Cover the tray and incubate the tray for 30 minutes at room temperature. It is advised to control the reaction on the plate regularly. In case of strong development the TMB reaction can be stopped sooner. Avoid exposing the microwell strips to direct sunlight. Covering the plate with aluminium foil is recommended.
16. Stop the reaction by adding 100 µl of stop solution with the same sequence and timing as used in step 14. Mix solutions in the wells thoroughly by gently swirling the plate. Gently tap the tray to eliminate any air bubbles trapped in the wells.
17. Read the plate within 30 minutes after addition of stop solution at 450 nm using a plate reader, following the instructions provided by the instrument's manufacturer.

*) In case plate washer is used, please note: use of a plate washer can result in higher background and decrease in sensitivity. We advise validation of the plate washer with the manual procedure.
Make sure the plate washer is used as specified for the manual method.

10. INTERPRETATION OF RESULTS

- Calculate the mean absorbance for each set of duplicate standards, control and samples.
- If individual absorbance values differ by more than 15% from the corresponding mean value, the result is considered suspect and the sample should be retested.
- The mean absorbance of the highest standard should be less than 0.35.
- Create a standard curve using computer software capable of generating a good curve fit. The mean absorbance for each standard concentration is plotted on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis (logarithmic scale).
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
- Samples that give a mean absorbance under the absorbance for the highest standard concentration are out of range of the assay. These samples should be retested at a higher dilution.

11. TECHNICAL HINTS

- User should be trained and familiar with ELISA assays and test procedure.
- If you are not familiar with the ELISA technique it is recommended to perform a pilot assay prior to evaluation of your samples. Perform the assay with a standard curve only following the instructions.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing wash buffer, fill with wash buffer as indicated for each cycle and do not allow wells to sit uncovered or dry for extended periods.
- Since exact conditions may vary from assay to assay, a standard curve must be established for every run. Samples should be referred to the standard curve prepared on the same plate.
- Do not mix reagents from different batches, or other reagents and strips. Remainders should not be mixed with contents of freshly opened vials.
- Each time the kit is used, fresh dilutions of standard, sample, tracer, streptavidin-peroxidase and buffers should be made.
- Caps and vials are not interchangeable. Caps should be replaced on the corresponding vials.
- To avoid cross-contaminations, change pipette tips between reagent additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- The waste disposal should be performed according to your laboratory regulations.

Technical support

Do not hesitate to contact our technical support team at support@hycultbiotech.com for inquiries and technical support regarding the Competitive Nitrotyrosine ELISA.

Hycult Biotech, Frontstraat 2a, 5405 PB Uden, the Netherlands

T: +31 (0)413 251 335, F: +31 (0)413 248 353

12. QUALITY CONTROL

The Certificate of Analysis included in this kit is lot specific and is to be used to verify results obtained by your laboratory. The absorption values provided on the Certificate of Analysis are to be used as a guideline only. The results obtained by your laboratory may differ.

This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Hycult Biotech immunoassay, the possibility of interference cannot be excluded.

For optimal performance of this kit, it is advised to work according to good laboratory practice.

13. TROUBLESHOOTING

Warranty claims and complaints in respect of deficiencies must be logged before expiry date of the product. A written complaint containing lot number of the product and experimental data shall be sent to support@hycultbiotech.com.

Suggestions summarized below in Table 3 can be used as guideline in case of unexpected assay results.

Low absorbance	High absorbance	Poor duplicates	All wells positive	All wells negative	Possible cause
•	•		•	•	Kit materials or reagents are contaminated or expired
•					Incorrect reagents used
•		•	•	•	Lyophilized reagents are not properly reconstituted
•	•	•	•	•	Incorrect dilutions or pipetting errors
•	•	•	•	•	Improper plastics used for preparation of standard and/or samples
•	•				Improper incubation times or temperature
		•			Especially in case of 37°C incubation: plates are not incubated uniformly
•					Assay performed before reagents were adapted to room temperature
•	•	•	•	•	Procedure not followed correctly
			•	•	Omission of a reagent or a step
		•			Poor mixing of samples
	•		•		Low purity of water
	•	•			Strips were kept dry for too long during/after washing
•		•	•		Inefficient washing
•	•	•			Cross-contamination from other samples or positive control
		•	•		TMB solution is not clear or colorless
•	•				Wrong filter in the microtiter reader
	•	•			Airbubbles
		•			Imprecise sealing of the plate after use
•					Wrong storage conditions
•					Lamp in microplate reader is not functioning optimally

Table 3