

Version 3 Last updated 16 January 2019

ab116691

3-Nitrotyrosine ELISA Kit

For the quantitative measurement of 3-nitrotyrosine modified proteins.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Principle: ab116691 3-nitrotyrosine ELISA (Enzyme-Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of 3-nitrotyrosine in cell and tissue lysates. The assay employs an antibody specific for 3-nitrotyrosine coated on a 96-well plate. Standards and samples are pipetted into the wells and 3-nitrotyrosine present in the sample is bound to the wells by the immobilized antibody. The wells are washed and a biotin labeled anti-3-nitrotyrosine detector antibody is added. After washing away unbound detector antibody, HRP-conjugated streptavidin specific for the biotin labeled detector antibody is pipetted into the wells. The wells are again washed, an HRP substrate solution (TMB) is added to the wells and color develops in proportion to the amount of 3-nitrotyrosine bound. The developing blue color is measured at 600 nm. Optionally the reaction can be stopped by adding hydrochloric acid which changes the color from blue to yellow and the intensity can be measured at 450 nm.

Background: 3-nitrotyrosine modification of proteins is a well established marker of protein damage by oxidative stress. 3-nitrotyrosine is a product of protein tyrosine nitration resulting from oxidative damage to proteins by peroxynitrite. Peroxynitrite is formed in vivo by the reaction of nitric oxide, a cellular messenger, and superoxide, the majority of which is generated by the mitochondrial respiratory chain. 3-nitrotyrosine modification of proteins can result in changes in protein structure, function and catalytic activity. Tyrosine nitration may increase (e.g. sGC, Src, PI3K, Akt), decrease (e.g. Mn-SOD, Ca⁺⁺-ATPase), or have no discernable effect (e.g. p53, VASP, α -Synuclein) on the activity of a particular protein. Tyrosine nitration has been implicated in the pathogenesis of major neurological (Alzheimer's, Parkinson's, multiple sclerosis, and stroke) and cardiovascular (atherosclerosis, myocardial infarction, coronary artery disease, hypertension) diseases that share inflammation as a contributor to pathogenesis.

2. Protocol Summary

Bring all reagents to room temperature. Prepare all the reagents, samples, and standards as instructed.



Add 50 μ L standard or sample to each well used. Incubate 2 hours at room temperature.



Aspirate and wash each well two times. Add 50 μ L prepared detector antibody to each well. Incubate 1 hour at room temperature.



Aspirate and wash each well two times. Add 50 μ L prepared HRP label. Incubate 1 hour at room temperature.



Aspirate and wash each well three times. Add 100 μ L HRP Development Solution to each well. Record immediately the color development with time at 600 nm for 15 minutes.

Alternatively add a Stop solution at a user-defined time and read at 450 nm.

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at +4°C immediately upon receipt. Kit has a storage time of 6 months from receipt, providing components have not been reconstituted.

After reconstitution, the standard should be stored at -80°C.

Unused microplate strips should be returned to the pouch containing the desiccant and resealed.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage Condition
20X Buffer	20 mL	4°C
Extraction Buffer	15 mL	4°C
10X Blocking Buffer	6 mL	4°C
HRP Development Solution	12 mL	4°C
10X 3-nitrotyrosine Detector Antibody	1 mL	4°C
10X HRP Label	1 mL	4°C
3-nitrotyrosine BSA standard (4 µg)	1 vial	4°C
Microplate 96 antibody coated wells in 12 strips	1 unit	4°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at 600 nm (or 450 nm after addition of Stop solution - not supplied).
- Method for determining protein concentration (BCA assay recommended).
- Deionized water
- Multi and single channel pipettes
- PBS (1.4 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 140 mM NaCl , 2.7 mM KCl , pH 7.3)
- Tubes for standard dilution
- Stop solution (optional) – 1N hydrochloric acid
- Optional plate shaker for all incubation steps

8. Technical Hints

- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells.
 - Prepare only as much reagent as is needed on the day of the experiment.
-
- 9.1 Bring all reagents and samples to room temperature (18-25°C) before use.
 - 9.2 Prepare 1X Wash Buffer by adding 20 mL 20X Buffer to 380 mL nanopure water.
 - 9.3 Prepare 1X Incubation Buffer by adding 6 mL 10X Blocking Buffer to 54 mL 1X Wash Buffer. After performing the ELISA freeze unused 1X Incubation buffer.
 - 9.4 Dilute the 3-nitrotyrosine protein detector antibody 10-fold with 1X Incubation Buffer immediately before use. Prepare 0.5 mL for each strip used.
 - 9.5 Dilute the HRP label 10-fold with 1X Incubation Buffer immediately before use. Prepare 0.5 mL for each strip used.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).

- 10.1** Reconstitute the standard with 1 mL 1X Incubation buffer by pipetting. Allow to sit for 10 minutes and repeat pipetting to ensure thorough reconstitution. This 4 µg/mL stock of standard material is then used to generate a standard curve in labeled tubes. Add 150 µL of the stock to a tube labeled #1 dilute Tube #1 further by adding 3 volumes, 450 µL, of 1X incubation buffer for a final concentration of 1 µg/mL. The remaining stock material can be stored at -80°C.
- 10.2** Label tubes #2-7. Add 150 µL of 1X Incubation buffer to each of #2 through #15. Transfer 150 µL from 1 µg/mL Tube #1 to tube #2. Mix thoroughly. With a fresh pipette tip transfer 150 µL from #2 to #3. Mix thoroughly. Repeat for Tubes #4 through #7. Use 1X Incubation buffer as the zero standard. Use a fresh standard for each assay.

11. Sample Preparation

ΔNote: Extraction buffer can be supplemented with phosphatase inhibitors, PMSF and protease inhibitor cocktail prior to use. Supplements should be used according to manufacturer's instructions.

11.1 Cell Lysates:

Collect non-adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 g for 10 min at 4°C. Rinse cells twice with PBS. Solubilize cell pellet at 2×10^7 /mL in Extraction Buffer. Incubate on ice for 20 minutes. Centrifuge at $16000 \times g$ 4°C for 20 minutes. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.2 Tissue Lysates:

Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended). Suspend the homogenate to 25 mg/mL in PBS. Solubilize the homogenate by adding 4 volumes of Extraction Buffer to a sample protein concentration of 5 mg/mL. Incubate on ice for 20 minutes. Centrifuge at $16000 \times g$ 4°C for 20 minutes. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

The sample should be diluted to within the working range of the assay in 1X Incubation Buffer.

As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Typical Sample Values.

Refer to Dilution Guidelines for further instruction.

Guidelines for Dilutions of 100-fold or Greater <i>(for reference only; please follow the insert for specific dilution suggested)</i>	
100x	10000x
4 µl sample + 396 µl buffer (100X) = 100-fold dilution <i>Assuming the needed volume is less than or equal to 400 µl</i>	A) 4 µl sample + 396 µl buffer (100X) B) 4 µl of A + 396 µl buffer (100X) = 10000-fold dilution <i>Assuming the needed volume is less than or equal to 400 µl</i>
1000x	100000x
A) 4 µl sample + 396 µl buffer (100X) B) 24 µl of A + 216 µl buffer (10X) = 1000-fold dilution <i>Assuming the needed volume is less than or equal to 240 µl</i>	A) 4 µl sample + 396 µl buffer (100X) B) 4 µl of A + 396 µl buffer (100X) C) 24 µl of A + 216 µl buffer (10X) = 100000-fold dilution <i>Assuming the needed volume is less than or equal to 240 µl</i>

12. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
 - We recommend that you assay all standards, controls and samples in duplicate.
-
- 12.1 Prepare all reagents, working standards, and samples as directed in the previous sections.
 - 12.2 Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
 - 12.3 Add 50 μ L of each diluted Standard or sample per well. It is recommended to include a dilution series of a control (normal) sample as a reference. Also include a 1X Incubation buffer as a zero standard.
 - 12.4 Cover/seal the plate and incubate for 2 hours at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.
 - 12.5 Aspirate each well and wash, repeat this once more for a total of two washes. Wash by aspirating or decanting from wells then dispensing 300 μ L 1X Wash buffer into each well as described above. Complete removal of liquid at each step is essential to good performance. After the last wash, remove the remaining buffer by aspiration or decanting. Invert the plate and blot it against clean paper towels to remove excess liquid.
 - 12.6 Immediately before use prepare sufficient (0.5 mL/strip used) 1X Detector Antibody in 1X Incubation buffer. Add 50 μ L 1X Detector antibody to each well used. Cover/seal the plate and incubate for 1 hour at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.
 - 12.7 Repeat the aspirate/wash procedure above.
 - 12.8 Immediately before use prepare sufficient (0.5 mL/strip used) 1X HRP label in 1X Incubation buffer. Add 50 μ L 1X HRP label to each well used. Cover/seal the plate and incubate for 1 hour at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.
 - 12.9 Repeat the aspirate/wash procedure above, however, performing a total of three washes.

12.10 Add 100 μ L HRP Development Solution to each empty well and immediately record the blue color development with time in the microplate reader prepared with the following settings:

Mode	Kinetic
Wavelength	600 nm
Time	Up to 15 minutes
Interval	20 – 60 seconds
Shaking	Shake between readings

Alternative– In place of a kinetic reading, at a **user defined**, time record the endpoint OD data at (i) 600 nm or (ii) stop the reaction by adding 100 μ L stop solution (1N HCl) to each well and record the OD at 450 nm.

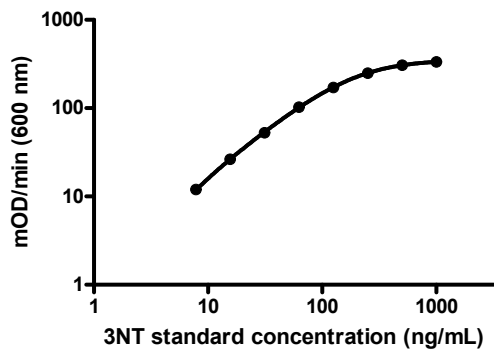
12.11 Analyze the data as described below.

13. Calculations

Average the duplicate standard readings and plot against their concentrations after subtracting the zero standard reading. Draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A four-parameter algorithm (4PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4-parameter logistic). Read 3-nitrotyrosine BSA standard equivalents for unknown and control samples from the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted in 1X Incubation buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

14. Typical Data

Typical standard curve – data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.



Standard (ng/mL)	Change mOD/min (600nm)
8	13, 11
16	26, 27
31	51, 55
62	100, 106
125	167, 177
250	234, 255
500	303, 309
1000	332, 335

Figure 1. Example Standard Curve.

15. Typical Sample Values

MOLARITY CORRECTION FACTOR –

The provided 4000 ng/mL undiluted standard is a 3NT labeled BSA sample (MW = 66.7 kDa), the concentration of this is therefore 60 nM. It was determined spectrophotometrically that there are 7 nitrotyrosine residues per BSA molecule therefore the concentration of 3NT in this sample is 420 nM. This means there are 0.105 nM in the sample for every 1 ng/mL. Results can be converted to nM by this 0.105X correction factor.

SENSITIVITY –

Typical sensitivity and working range = 8 - 1000 ng/mL.

3NT modified BSA standard = 0.84 - 105 nM.

PRECISION –

	Intra-assay Precision	Inter-Assay Precision
CV (%)	6.9 (n=8)	13 (n=3)

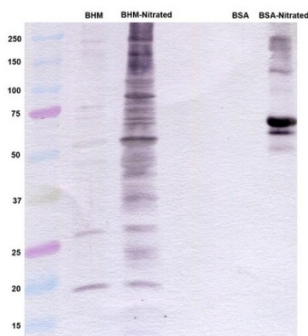
Linearity of Dilution

Sample Type	% Expected
1:1	100
1:2	115
1:4	99
1:8	79
1:16	80
1:32	78
1:64	90

16. Assay Specificity

Species – all.

The antibody used in this kit is available as individual antibody ab110282.



ab110282 identifies nitrated samples. Bovine heart mitochondria and BSA were nitrated and run alongside non-nitrated samples. ab110282 showed specificity to the nitrated samples.

Please contact our Technical Support team for more information.

17.Troubleshooting

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standard dilution	Ensure briefly spin the vial of Item C and dissolve the powder thoroughly by a gentle mix.
Low signal	Too brief incubation times	Ensure sufficient incubation time; standard/sample change incubation to over night
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your standard at -80°C after reconstitution, others at 4°C. Keep substrate solution protected from light

18. Notes

Technical Support

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