Version 3 Last updated 23 January 2019

ab113848
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

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 a 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, a-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.

Principles: ab113848 is used to determine the level of nitrotyrosine modified proteins in a sample. The provided microplates have been evenly coated with a nitrotyrosine containing antigen. The competitive ELISA is performed by adding the test sample mixed with the provided HRP conjugated anti-3NT antibody. If no 3NT modified proteins are present in the sample, all of the anti-3NT detector antibody is available to bind to the immobilized 3NT containing protein coating the wells, generating a maximum (100%) binding signal. In contrast, if soluble 3NT modified protein is present in the sample, it will compete for binding by the anti-3NT detector antibody. The degree of competition is proportional to the concentration of soluble 3NT modified proteins in the sample. Therefore, the signal in each well has an inverse relationship to the amount of 3NT in each sample. When performing each assay, a standard curve is generated from the provided standard to allow the accurate quantitation of the 3NT content in the test samples.

The assay is followed by monitoring the HRP-dependent color change in each well at 600 nm. Alternatively, the assay can be terminated, at a user-defined time, by the addition of 1N HCl (not supplied) and the assay performed as an end-point measurement at 450 nm

2. Protocol Summary

Bring all reagents to room temperature.

Prepare all the reagents, samples, and standards as instructed.

Add 50 µL 2X standard or sample to each well used.

Add 50 µL 2X HRP detector antibody to each well used.

Incubate 2 hours at room temperature.

Aspirate and wash each well four times.

Add 100 µL TMB One-Step Substrate Reagent to each well.

Record immediately the color development with time at 600 nm for 15 minutes.

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 dessicant 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
3NT BSA Standard 4 µg	1 vial	4°C
Extraction Buffer	15 mL	4°C
20X Wash Buffer	25 mL	4°C
10X Blocking Solution	10 mL	4°C
1000X HRP-conjugated 3NT Detector Antibody	0.04 mL	4°C
1X Development Solution	24 mL	4°C
Nitrotyrosine coated 96-well Solid Plate	2 units	4°C
Plate Seals	2 units	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

- To avoid cross contamination, change pipette tips between additions of each standard, sample and between reagent additions. Also use separate clean, dry reservoirs for each reagent.
- Cover plate during incubation steps.
- Thorough and consistent wash technique is essential for proper assay performance. Wash buffer must be forcefully dispensed and completely removed from the wells by aspiration or decanting. Remove remaining wash buffer by inverting the plate and blotting on paper towels.
- HRP development solution should remain colorless until added to the plate. Keep HRP development solution protected from light. HRP development solution should change color from colorless to blue.
- If using Stop solution, this should be added to the plate in the same order and timing as the HRP development solution. The color developed in the wells should tune from blue to yellow with gentle mixing.

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 Prepare 1X Wash Buffer by adding 25 mL 20X Wash Buffer to 475 mL nanopure water.

9.2 Prepare 2X Incubation Buffer by adding 10 mL 10X Blocking Buffer to 40 mL 1X Wash Buffer. After performing the ELISA freeze unused 2X Incubation buffer.

9.3 Immediately before use prepare 2X HRP-Detector antibody. For an entire plate add 12 µL 1000X Detector antibody to 6mL 2X Incubation Buffer. Otherwise 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 Wash Buffer by pipetting. Allow to sit for 10 minutes and repeat pipetting to ensure thorough reconstitution. The stock of 3NT standard is now 4000 ng/mL. This stock of standard material is then used to generate a standard curve in labelled tubes as described below. Any remaining stock material can be stored at -80°C.

10.2 To create a 2-fold standard curve label tubes #1-7. Transfer 600 µL from stock to tube #1. Add 300 µL of 1X Wash Buffer to each of #2 through #7. Transfer 300 µL from stock tube #1 to tube #2. Mix thoroughly. With a fresh pipette tip transfer 300 µL from #2 to #3. Mix thoroughly. Repeat for Tubes #4 through #7. Use 1X Wash buffer as the zero standard tube #8. Any remaining stock material can be stored at -80°C.

11. Sample Preparation

Cellular samples must be detergent extracted by the provided Extraction buffer as described below. For serum and other tissue fluids, extraction may not be necessary.

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 $2x10^7$/mL in 1X sample extraction buffer. Incubate on ice for 20 minutes. Centrifuge at 12000 x 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 1X sample extraction buffer to a sample protein concentration of 5 mg/mL. Incubate on ice for 20 minutes. Centrifuge at 12000 x 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.

11.3 Sub-cellular organelle lysates (e.g. mitochondria):

Prepare the organelle sample by, for example, sub-cellular fractionation. Pellet the sample. Solubilize the pellet by adding 9 volumes 1X sample extraction buffer. Incubate on ice for 20 minutes. Centrifuge at 12000 x 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. These test samples should be diluted to within the working range of the assay in 1X Wash Buffer.

Refer to Dilution Guidelines for further instruction.

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

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 real.

12.3 Add 50 µL of each diluted Standards per well. It is recommended to use a plate map to record the location of standards. Unused standards can be frozen at -80°C.

12.4 Test samples should also be prepared and diluted in 1X Wash Buffer as described above. A dilution series of samples is recommended to ensure that samples are within the working range of the assay. Add 50 µL of each diluted sample into individual wells. It is recommended to use a plate map to record the location of test samples. Unused test samples can be frozen at -80°C.

12.5 Add 50 µL of 2X HRP Detector Antibody into each well of the plate used (for both standard and test samples). Note both the standard and test samples have been diluted 2X by this addition.

12.6 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.

12.7 Aspirate each well and wash, repeat this three more times for a total of four washes. Wash by aspirating or decanting from wells then dispensing 300 µL 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.

12.8 Add 100 µL 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	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 µL stop solution (1N HCl) to each well and record the OD at 450 nm. When using stop solution do not allow the blue color development to proceed for too long, intense blue coloration can lead to saturating conditions at 450nm.

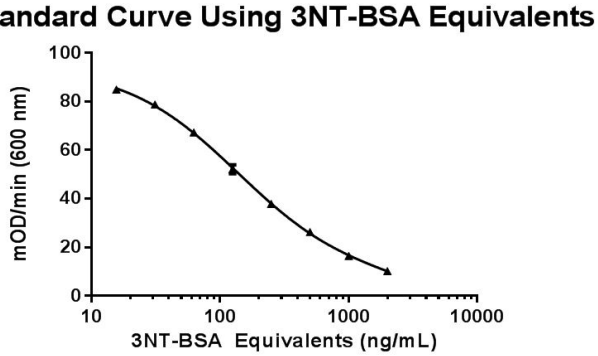
Analyze the data as described below.

13. Calculations

Average the duplicate standard readings and plot against their concentrations. 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 fou- 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 3NT concentrations 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 Wash buffer and reanalyzed, 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)	Average Change mOD/min (600nm)
2000	10.18
1000	16.44
500	26.31
250	37.91
125	52.08
62.5	67.27
31.25	78.81
15.63	84.97

Figure 1. Example standard curve in 3NT BSA equivalents.

Representing Data as Molarity

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 resides per BSA molecule therefore the concentration of 3NT in this sample is 4.2x10-7M. When the standards are mixed in the well with an equal volume of detector antibody the concentration is reduced by half, therefore the final concentration of the highest standard, 2000 ng/mL, is $2.1x10^{-7}$M.

Final concentration and molarity of example dilution series:

Well	1	2	3	4	5	6	7	8
Final concentration ng/mL	2000	1000	500	250	125	62.5	31	16
Final 3NT molarity	2.1 $x10^{-7}$	1.1 $x10^{-7}$	5.3 $x10^{-8}$	2.6 $x10^{-8}$	1.3 $x10^{-8}$	6.6 $x10^{-9}$	3.3 $x10^{-9}$	1.6 $x10^{-9}$

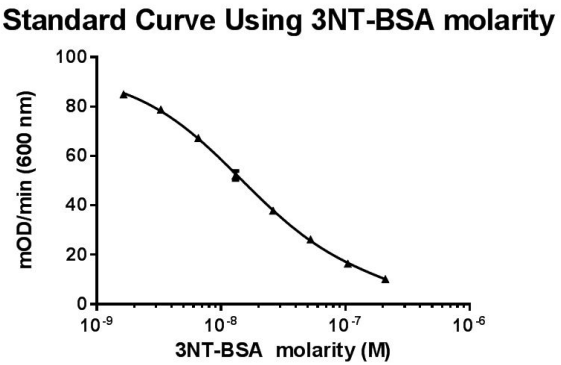


Figure 2. The same example standard curve in 3NT molarity.

15. Typical Sample Values

SENSITIVITY –

Minimum detectable dose = 10 ng 3NT-BSA standard

(i.e. 1x10-9 M 3NT). Calculated from interpolation of zero standard + 2 standard deviation.

3NT-BSA equivalents: 10 - 2000 ng/mL.

3NT molarity: 1x10-9 - 2x10-7 M.

PRECISION –

	Intra-assay Precision (n=8)	Inter-Assay Precision (n=40)
CV (%)	< 4	< 6
Range (%)	1 - 9.4	2.5 - 8.2

Linearity of Dilution

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Plasma and serum samples were serially-diluted to test for linearity.

A 3NT modified sample of GAPDH was analyzed using this competitive ELISA. A dilution series of 6 concentrations of GAPDH were measured in duplicate according to the above protocol. There are two ways to quantify and report the results.

Report 3NT-BSA equivalents

Well	3NT GAPDH final concentration ng/mL	Determined 3NT BSA equivalents ng/mL	GAPDH:3NT ng/ng	Linearity of dilution

1	1000	333	0.33	100%
2	500	200	0.39	118%
3	250	100	0.40	121%
4	125	42	0.34	103%
5	62.5	26	0.42	127%
6	31.25	11	0.35	106%

Conclusion: across 6 dilutions (a 32X dilution range) the 3NT modification of the GAPDH sample was determined to be 0.37 ng 3NT-BSA equivalents per ng GAPDH.

Report molarity (M) mol/L 3NT.

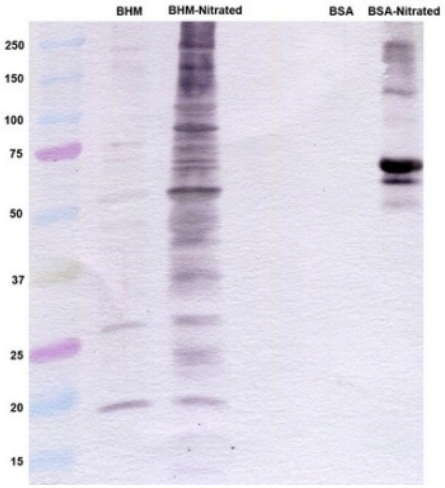
Well	Molarity of GAPDH (MW 35.8 kDa)	Molarity of 3NT	3NT:GAPDH
1	$2.8x10^{-8}$	$3.5x10^{-8}$	1.3
2	$1.4x10^{-8}$	$2.1x10^{-8}$	1.5
3	$6.9x10^{-9}$	$1.1x10^{-8}$	1.6
4	$3.4x10^{-9}$	$4.5x10^{-9}$	1.3
5	$1.7x10^{-9}$	$2.8x10^{-9}$	1.7
6	$8.6x10^{-10}$	$1.2x10^{-9}$	1.4

Conclusion: across 6 dilutions (a 32X dilution range), the 3NT modification of the GAPDH sample was determined to be 1.5 mol 3NT per mol GAPDH, i.e. an average of 1.5 nitrotyrosine resides per GAPDH protein.

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 (MS703) 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 standard and dissolve thoroughly by a gentle mix.
Low signal	Too brief incubation times	Ensure sufficient incubation time; standard/sample change incubation to overnight.
	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

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