# Nitrotyrosine Western blot starter pack

Cat no. **A010-513**

**A convenient reagent pack containing Badrilla's high integrity nitrotyrosine monoclonal antibody (clone 2E11) and associated positive and negative controls. Pack contains:**

1. Detailed Western blotting protocol
2. Nitrotyrosine monoclonal antibody (clone 2E11)
3. Nitrated albumin (positive control)
4. BSA (negative control)

Please read this manual completely prior to using the product.

**FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES**

## Pack components

| Reagent | Quantity | Storage |
|---|---|---|
| Detailed Western blotting protocol | | |
| Nitrotyrosine mAb (clone 2E11) | 50µg | -20ºC |
| NY-BSA (positive control) | 100µg | 4ºC |
| BSA (negative control) | 100µg | 4ºC |

## Protocol: Detection of 3-nitrotyrosine in protein samples by Western blotting

| Step | Action |
|---|---|
| **1** | Prepare samples: +ve control (A010-NYBSA), -ve control (A010-BSA) by addition of an appropriate volume of water (100µL) to create a concentration of 1mg/ml. Prepare biological samples consistent with the objectives of your research plan. |
| **2** | Mix samples with an apropriate volume of 2- or 5-strength Laemmli sample buffer, to achieve a protein concentration of 0.1-1.0mg/ml and a Laemmli sample buffer composition of 1-strength (62.5mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.05% bromophenol blue, 5% β-mercaptoethanol).<br><br>Optimal concentration of +ve and –ve control samples is 0.1mg/ml, optimal concentration of cell/tissue samples is 1mg/ml. |
| **3** | Incubate samples at 30C for 30 minutes, or at 100ºC for 5 minutes. |
| **4** | Load samples (1-10µg for +ve and -ve controls; 10-50µg for biological samples, typically). Separate proteins by SDS-PAGE to achieve optimal resolution of components under study. Control samples have an apparent molecular weight of 68kDa, suitable SDS-PAGE gels range from 8%-15% acrylamide (37.5:1 acrylamide:bis-acrylamide).<br><br>10-12% acrylamide is preferred for proteins of 30-120kDa. |
| **5** | Transfer proteins to blotting membrane (PVDF (recommended); or nitrocellulose, nylon) by wet transfer process (Towbin *et al*., 1979: PNAS 76, 4350-4). Alternative transfer approaches can be used.<br><br>• Incubate SDS-PAGE in transfer buffer (composition) for 30 minutes before assembly into transfer apparatus<br>• Incubate PVDF membrane with methanol for 5 minutes, then incubate in |

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| | transfer buffer for 30 minutes before assembly into transfer apparatus<br>• Remove all air bubbles from each layer of the transfer "sandwich"<br>• Transfer overnight at 4ºC (settings) |
| **6** | Remove PVDF membrane from transfer apparatus and incubate in TBS-Tween-20-milk (50mM Tris-HCl pH 7.4, 0.9%w/v NaCl, 0.1%v/v Tween-20, 5% w/v milk powder proteins) for 30 minutes at room temperature (18-22ºC), with constant gentle agitation (on rotary shaker). Discard SDS-PAGE gel and other materials as directed by local waste handling policies. |
| **7** | Incubate PVDF membrane with primary antibody (A010-2E11-3D3: mouse monoclonal IgG2b) in TBS-Tween-20-milk (above) at a dilution of 1:2000 (or other, as optimised in your experimental system) for 3 hours at room temperature.<br><br>Minimise the volume of solutions used in this step to minimise the consumption of valuable reagents; mini-gel blot can be exposed to 2ml of solution using roller tube approach or sealing in plastic bag. |
| **8** | Remove primary antibody and wash PVDF membrane 5 times with fixed volume of TBS-Tween-20 (as above but without milk component), 5 minutes with constant (rotary) agitation.<br><br>• Volume of wash buffer differs with dish dimensions: recommend a buffer depth of 10mm<br>• Multiple PVDF membranes can be washed in single dish, provided they do not stick together during washing<br>• Measure all attributes of this (and all) step (time, volume, vessel, agitation parameters) to improve the reproducibility of data |
| **9** | Incubate PVDF with appropriately labelled secondary antibody (anti-mouse IgG) in TBS-Tween-20 at the working dilution optimised in your laboratory for 90 minutes at room temperature with constant agitation.<br><br>We use peroxidase conjugated secondary antibodies for robust, sensitive western blotting. |
| **10** | Remove secondary antibody and wash PVDF membrane once for 15 minutes and 5 times with fixed volume of TBS-Tween-20 (as above but without milk component), 5 minutes with constant (rotary) agitation.<br><br>• Volume of wash buffer differs with dish dimensions: recommend a buffer depth of 10mm.<br>• Multiple PVDF membranes can be washed in single dish, provided they do not stick together during washing.<br><br>Measure all attributes of this (and all) step (time, volume, vessel, agitation parameters) to improve the reproducibility of data. |

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| **11** | Incubate PVDF with substrate for detection of secondary antibody as required.<br><br>For peroxidase-based detection chemiluminescence substrates & detection are sensitive and robust. |
| **12** | Image the signal on PVDF membrane by CCD camera (recommended), or X-ray film (for chemiluminescence).<br><br>• Use exposures of variable durations (30, 60, 300 seconds, as appropriate)<br>• Avoid over exposure of signal, where intensity no longer reflects rate of signal production (related to biomarker abundance)<br>• Store data in all appropriate file formats<br>• Other detection systems will be needed where chemiluminescence is not the output (e.g. fluorescent dyes, radionucleides, chromogenic substrates). |

## Nitrotyrosine mAb (Clone 2E11) antibody

Quality Control Certificate of Analysis
Catalogue No.: A010-2E11
Unit Size: 50 µg
Lot No: 1211-01

**Background**: A large number of physiological and pathological events lead to so called stress conditions to which the organism can adapt within certain limits by regulating the activity of a series of enzymatic cascades. Most stress responses are characterized by an increased generation of “free radicals” which encompass both reactive oxygen (ROS) and nitrogen species (RNS). Most of these species react with macromolecules of the organism, i.e. amino acids, proteins, lipids and DNA, leading to functional alterations which can either participate to adaptation or lead to cell death. Oxidative stress however, has different functional consequences depending not only upon its intensity and duration, but also upon the nature of the free radicals, ROS or RNS, generated. Interestingly, the nature of these reactive species depends on the ratio between the initially produced ROS, superoxide anion (O2.-), and nitric oxide (NO). Indeed, O2.- very rapidly reacts with NO and therefore, as long as the NO/ O2.- ratio is = 1, O2.- will therefore preferentially react with NO rather than with macromolecules, thus generating reactive nitrogen species (RNS): nitrosonium (NO+), N2O3 and peroxinitrite (ONOO-). These RNS induce posttranslational modifications: for NO+ and N2O3, S-nitrosation (Cys-SNO) and for ONOO- tyrosine nitration (Tyr-NO2), methionine sulfoxidation (Met-SOH) and thiol oxidation (RS-S-R). When the O2./ NO ratio becomes > 1, the O2.- and NO.2 ions and thereafter the OH. radicals cause irreversible oxidations and peroxidations of macromolecules which generally lead to cell death(1-4). The monoclonal antibodies have been selected for their high affinity and specificity towards Tyr-NO2 residues. They do not cross-react with Tyr or Tyr derivatives such as aminotyrosine, chlorotyrosine or phosphotyrosine, neither with nitroTrp which can also be generated in response to peroxynitrite. They also recognize Tyr-NO2 residues in various sequences as shown by their ability to recognize nitrated proteins including albumin, ovalbumin, insulin, hemoglobin, KLH and various cytoplasmic and mitochondrial proteins.

**Description**: Lyophilised Mouse monoclonal affinity purified antibody (A010-2E11AP) containing IgG2b antibody specific for Nitrotyrosine

**Immunogen**: Protein-bound nitrotyrosine

**Antibody Isotype**: IgG2b.

**Antibody Purity**: Protein A affinity purified.

**Vial Constituents:** Lyophilised A010-2E11 (20 µl) in Phosphate Buffered Saline 10 mM, NaCl 0.15 M (pH 7.4) Thimerosal 0,01% may be used as preservative

**Storage Instructions:** Lyophilised antibody is stable at 4 °C when stored with desiccant. Reconstitute lyophilised powder in 20 µl of 18 MΩ $H_2O$, aliquot and store frozen at -80 °C for 1 year. Avoid freeze - thaw cycles.

**Antibody specificity / Cross reactivity:** Nitrotyrosine either free or incorporated in proteins such as human serum nitro albumin, nitro haemoglobin and nitro insulin. All species

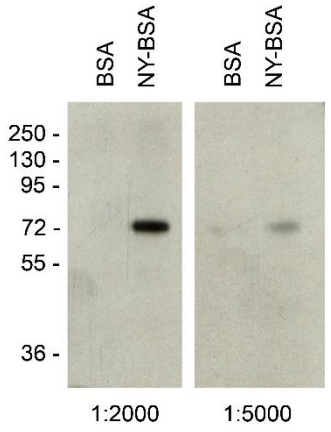


**Figure 1**

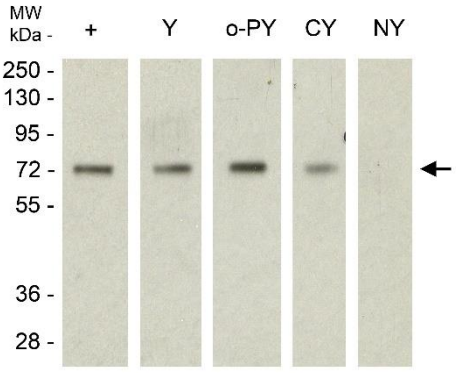


**Figure 2**

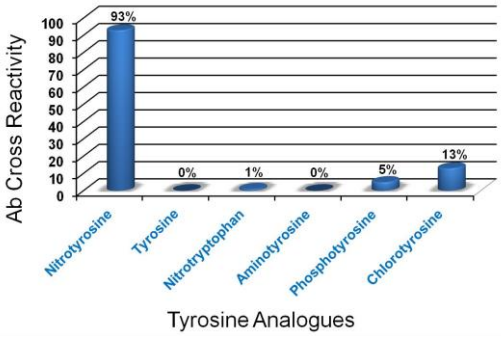


**Figure 3**

Figure 1: Western Blot using 1:2000 and 1:5000 Nitrotyrosine Clone 2E11-3D3 mouse monoclonal Ab (A010-2E11AP) against 10µg of BSA and NY-BSA. 10% gel, PVDF membrane

Figure 2: Western Blot using 1:2000 Clone 2E11-3D3 (A010-2E11AP) Ab against 10µg of NY-BSA +/- 100 µg/ml Tyrosine (Y), O-Phospho-L-Tyrosine (o-PY), 3-Chloro-L-Tyrosin (CY) and Nitrotyrosine (NY). 10% gel, PVDF membrane, 10 second film exposure. NY completely blocked Ab staining, CY caused some inhibition whereas Y and o-PY had no effect.

## Use of product

Unless otherwise stated in our catalogue or other company documentation accompanying the product(s), our products are intended for **Research Use Only** (RUO) and are not to be used for any other purpose, which includes, but is not limited to, unauthorised commercial uses, *in vitro* diagnostic uses, *ex vivo* or *in vivo* therapeutic uses or any type of consumption or application to humans or animals.

## Warranty

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