Product Manual

NITROSO-THIOL KIT

Catalog #: ALX-850-037-KI01

USE FOR RESEARCH PURPOSES ONLY

Unless otherwise specified expressly on the packaging, all products sold hereunder are intended for and may be used for research purposes only and may not be used for food, drug, cosmetic or household use or for the diagnosis or treatment of human beings. Purchase does not include any right or license to use, develop or otherwise exploit these products commercially. Any commercial use, development or exploitation of these products or development using these products without the express written authorization of Enzo Life Sciences, Inc. is strictly prohibited. Buyer assumes all risk and liability for the use and/or results obtained by the use of the products covered by this invoice whether used singularly or in combination with other products.

LIMITED WARRANTY; DISCLAIMER OF WARRANTIES

These products are offered under a limited warranty. The products are guaranteed to meet all appropriate specifications described in the package insert at the time of shipment. Enzo Life Sciences' sole obligation is to replace the product to the extent of the purchasing price. All claims must be made to Enzo Life Sciences, Inc., within five (5) days of receipt of order. THIS WARRANTY IS EXPRESSLY IN LIEU OF ANY OTHER WARRANTIES OR LIABILITIES, EXPRESS OR IMPLIED, INCLUDING WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, AND NON- INFRINGEMENT OF THE PATENT OR OTHER INTELLECTUAL PROPERTY RIGHTS OF OTHERS, AND ALL SUCH WARRANTIES (AND ANY OTHER WARRANTIES IMPLIED BY LAW) ARE EXPRESSLY DISCLAIMED.

TRADEMARKS AND PATENTS

Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending. Enzo is a trademark of Enzo Life Sciences, Inc.

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

Contents of the Kit:

Vial 1- Griess A + cleavage reagent
Vial 2- Griess A
Vial 3- Griess activator
Vial 4- Griess B
Vial 5- Nitroso-glutathione standard
Vial 5a- for diluting nitroso-glutathione standard
1 96 well plate
Kit Manual
If any item is missing, please email info-usa@enzolifesciences.com.

Important Storage requirements:

Due to a recent innovation, we can guarantee the performance of our kit for up to six months even when kept at room temperature. When stored at 4°C, the kit can be stored for up to one year. Whatever temperature is chosen, we do recommend, for optimum performance, that the nitroso-glutathione standard (Vial 5) is kept at -20°C in the dark.

Additional items required:

A plate reader with a 540 nm filter
An adjustable pipettor
A repeating pipettor (suggested)
High purity water- ideally MQ+ (R Waters Inc) or HPLC grade.

General tips for optimal performance:

The lower the quality of water used, the higher the background will be. Tap water, for example, contains nearly 1 mM nitrate. Dispose of pipette tip wastewater into a beaker: expelling waste into a sink can lead to splashback, which would result in significant contamination.

Sample number:

This kit is for 96 analyses. This includes standards, which **must be run at the same time** and **in the same plate as unknowns**. It is a matter of personal choice as to how many standards need to be run, however, we recommend a minimum of eight, run in duplicate. Thus, approximately 70-80 unknowns can be analyzed with one kit. Again, it is a matter of personal choice whether the unknowns are run in duplicate: if so then 35-40 unknowns can be analyzed with one kit.

Legal Strictures:

The kit is for research use only. It is not designed for clinical diagnostic purposes. Please take all appropriate care when opening the vials. We strongly recommend the use of eye protection and gloves.

Background:

The assay of nitrosothiols (RSNOs) appears to provide a very useful index of nitric oxide synthase (NOS) activity. The concentration of RSNOs has been shown to increase with localized induction of NOS. Further, they have been shown to fall rapidly upon administration of NOS inhibitors.

Our kit provides a complete solution for the assay of RSNOs for most fluid samples including cell lysates It is very flexible, allowing the researcher (by use of simple ultra filters), to assay low and/or high molecular weight RSNOs. The assay is divided into essentially two steps; in the first the sample is prepared according to which subgroup of RSNO is desired. The S-N bond is then cleaved, resulting in the formation of an equivalent of nitrite, which is assayed using the Griess reaction. The intensity of the color formed is linearly related to the concentration of nitrite and hence to the original RSNO concentration.

Sensitivity:

Sensitivity is determined by two factors: the fluid being analyzed and the detection method used. Plasma, for example, contains many substances that can interfere with the Griess Reaction. Consequently, only 40 μL of plasma may be analyzed in a well; sensitivity is therefore reduced by approximately 60%.

For all other fluids: using a plate reader, sensitivity is approximately 500 nM. However, if a microcuvette is used in a spectrophotometer, then up to a five-fold increase in sensitivity can be obtained.

Tips for Optimal Performance:

The most significant factors affecting the performance of this Kit are pipetting accuracy and background interference. We recommend the use of a repeating pipettor since this increases the precision of the delivery into the wells. Secondly, nitrate and nitrite are everywhere present; beakers, pipette tips, water, hands etc. Washing all plastic and glassware with high purity

water will significantly improve the results. In cell and tissue culture work, a very significant contaminant is the nitrite present in culture media. This results in a typical background of 2-3 μM nitrite. Our culture media contain approximately 400-600 nM nitrite-- a significant factor when one is trying to measure a typical 200 nM increase in RSNO/nitrite, after cell stimulation. Please call for ordering information.

ASSAY PROTOCOLS

Overview:

The chemistry of the Kit is based on two classical chemical reactions: Griess and Saville. In essence, a cleavage reaction breaks the S-N bond of nitroso-thiols. This results in the release of an equivalent of NO, which in turn gives an equivalent of nitrite. The nitrite is detected colorimetrically using the Griess reaction. It is a very precise and accurate method for measuring RSNO concentrations, however a potential problem is the presence of nitrite in your samples. For this reason, we include a second Griess A vial (Vial 2) which does **not** contain the cleavage reagent and therefore only detects free nitrite. One may simply subtract Vial 2 values from Vial 1 values to ascertain the contribution from RSNO. This is only necessary in situations where nitrite concentrations are expected to be a significant percentage of RSNO concentrations.

A typical analysis requires a group of wells from the 96 well plate to be used for a standard curve. In this Kit, we supply a known standard of nitroso-glutathione which may be added or spiked into replicate samples, or water if no spare sample is available. This will allow for the generation of a standard curve to determine the unknowns. We highly recommend that you run the standard curve in the bio-fluid you are analyzing. However, if this is not practical, you may dilute the GSNO into water as shown below. So, the first step is to set up the standard curve as follows:

How To Set Up The Nitroso-glutathione (GSNO) Standard curve.

Add **500 µL** (**NB_ This is a change from previous kit instructions**) of ultra pure water to Vial 5 and mix well; take 10 µL of this solution into 1.49 mL of water in the clean vial (Vial 5a) and mix well. This is your master solution, keep it at 4°C and wrapped in aluminum foil. Use ideally within 1-2 hours as it slowly decomposes when dissolved in water. Include at least seven standards from the following table in your sample set. Also, add 200 µL of water to two wells for use as blanks for the plate reader.

STEP 1: Generate a GSNO standard curve: Pipette out from Vial 5a as follows into wells designated as standard wells; then add ultra pure water as indicated in the second column:

µL Vial 5a	µL bio-fluid/water	Final concentration GSNO µM
0	100	0
2.5	97.5	0.25
5.0	95	0.5
10	90	1.0
20	80	2.0
30	70	3.0
40	60	4.0
60	40	6.0
100	0	10.0

Samples: For total (i.e. high and low MW) RSNO, simply add 100 µL of sample into wells designated for unknowns. Then immediately follow the Step 2 process outlined on the next page.

For high or low RSNOs, simply ultrafilter the sample using an ultra free filter (e.g. Millipore). Then, either transfer 100 µL of the filtrate to a well for low MW RSNOs or add water to the retentate so as to bring it back to the volume applied to the filter, and use 100 µL of that for high MW RSNOs. Once prepared, follow Step 2 on next page.

Once the plate is set up, proceed to Step 2 as quickly as possible.

NOTE: For plasma analysis, a slight modification is required. Firstly only 40 μL may be analyzed--dilute to 100 μL with ultra-pure water in the well. Further, older or multiply frozen plasma tends to give rise to colloidal samples during the analysis, which artificially increases absorbency reading. To reduce this effect, spin the samples at 5000 g for 10 minutes prior to analysis. Better, use an HPLC type 0.22 μm filter to remove particulate matter. This effect is rarely seen in fresh plasma.

STEP 2 - Griess Assay for Nitroso-thiol

Prepare Griess Reagents:

Add 0.4 mL from Vial 3 to Vial 1, and 0.4 mL from Vial 3 to Vial 2. Gently mix well and with care! Store on ice. **N.B. Some precipitation may be present in Vials 1, 2 or 4; this is normal and in no way affects the performance of the kit.**

Add Color Generating Reagents (Griess)

Add 50 μL from Vial 1 to each well, followed immediately by 50 μL from Vial 4 to each well. A purple color should be visible. Wait 10 minutes, zero the plate reader against the water-only blanks and then read the plate at 540 nm to obtain the absorbency values for each well.

Calculate the results

Plot the absorbency of the known standards against concentration; this will be a straight line. The concentration of the unknowns can then be read off the standard curve.

Control Reagent (Vial 2):

Since high background levels of nitrite in the samples will give rise to color formation in addition to those of nitrosothiol concentration, we supply Vial 2 which is Griess A but without the cleavage reagent. Should you wish to check that the reading you are getting is truly derived from a nitrosothiol, simply re-analyze the sample in duplicate. To one add 50 μL of Vial 1 as before, and to the other instead add 50 μL of Vial 2. The difference between the first and second sample will indicate the amount of signal deriving exclusively from RSNO. If the changes in RSNO

are expected to be small relative to nitrite concentrations, you may wish to analyze all your samples in this way.

Potential problems:

If you experience any difficulty please try the following remedies. If, however, you are still having problems, please email our Technical Support Team.

1. Poor Precision:

It is undoubtedly due to poor pipetting technique or particulates/bubbles in the wells. Try using a repeating pipette for optimal accuracy. In our studies, the kit assay has a very low cov (<5% in the mid-range of the standard curve). Also, try coating each tip with the reagent or sample to be pipetted- i.e. take up the required volume and expel back into the vial, repeat and then dispense the required volume into the well. This really helps precision.

2. No color!

Have you added the color reagents? (Vials 1 & 4). If any of the reagents were omitted, you unfortunately will have to repeat the whole analysis.

3. High Background?

Check a few of your samples with Vial 2 as detailed above-- you may have high concentrations of nitrite in your samples.

3. Using Cell Lysates?

The reagents used in this kit will not permeate cells. If using epithelial cells that adhere to the plate, simply remove the buffer/medium they are growing in and replace with distilled water which will lyse the cells. The water can then be removed either by filtration or spinning, and then the lysate can be used in the assay. If using frozen lysates, simply spin/filter after thawing and use the kit to assay the NOthiols

Notes:

Notes:

Notes:

Product Manual

www.enzolifesciences.com

Enabling Discovery in Life Science®

GLOBAL HEADQUARTERS
Enzo Life Sciences Inc.
10 Executive Boulevard
Farmingdale, NY 11735
Toll-Free:1.800.942.0430
Phone:631.694.7070
Fax: 631.694.7501
info-usa@enzolifesciences.com

EUROPE/ASIA
Enzo Life Sciences (ELS) AG
Industriestrasse 17
CH-4415 Lausen
Switzerland
Phone:+41/0 61 926 89 89
Fax:+41/0 61 926 89 79
info-ch@enzolifesciences.com

For local distributors and detailed product information visit us online:
www.enzolifesciences.com

Catalog Number: ALX-850-037 Rev. 11/13/17