NO Detection Kit

for fluorescence microscopy

Instruction Manual

Cat. No. ENZ-51013-200 200 fluorescence microscopy assays

For research use only.

Rev. 1.0 April 2009

Notice to Purchaser

The NO Detection Kit is a member of the CELLestial™ product line, reagents and assay kits comprising fluorescent molecular probes that have been extensively benchmarked for live cell analysis applications. CELLestial™ reagents and kits are optimal for use in demanding imaging applications, such as confocal microscopy, flow cytometry and HCS, where consistency and reproducibility are required.

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I. Introduction

Free radicals and other reactive species play influential roles in many human physiological and pathophysiological processes, including cel signaling, aging, cancer, atherosclerosis, macular degeneration, sepsis, various neurodegenerative diseases (Alzheimer's and Parkinson's disease) and diabetes. Once produced within a cell, free radicals can damage a wide variety of cellular constituents, including proteins, lipids and DNA. However, at lower concentrations these very same agents may serve as second messengers in cellular signaling. Information-rich methods are required to quantify the relative levels of various reactive species in living cells and tissues, due to the seminal role they play in physiology and pathophysiology. The **NO Detection Kit** provides a simple and specific assay for the real-time measurement of free nitric oxide (NO) and by extension nitric oxide synthase (NOS) activity in living cells.

This kit is designed to directly monitor real time NO production in live cells by fluorescence microscopy using NO Detection Reagent (red fluorescent) as the major component. The non-fluorescent, cell-permeable NO detection dye reacts with NO in the presence of O_2 with high specificity, sensitivity and accuracy, yielding a water-insoluble red fluorescent product. Importantly, this dye is not reactive towards peroxynitrite that allows distinguishing between peroxynitrite and nitric oxide. The kit is not designed to detect reactive chlorine or bromine species, as the fluorescent probe included is relatively insensitive to these analytes. Upon staining, the fluorescent products generated by this dye can be visualized using a wide-field fluorescence microscope equipped with any red fluorescent cube. The combination of 650/670 nm is recommended when additional fluorescence signals (green or orange) would be detected simultaneously.

II. Reagents Provided and Storage

All reagents are shipped on dry ice. Upon receipt, the kit should be stored at -20°C, or -80°C for long term storage. When stored properly, these reagents are stable for at least twelve months. **Avoid repeated freezing and thawing.**

Reagents provided in the kit are sufficient for at least 200 fluorescence microscopy assays using live cells (adherent or in suspension).

Reagent	Quantity
NO Detection Reagent (Red)	60 µL
NO Inducer (L-Arginine)	100 µL
NO Scavenger (c-PTIO)	400 nmoles
10X Wash Buffer	15 mL

III. Additional Materials Required

- CO_2 incubator (37°C)
- Standard fluorescence microscope
- Calibrated, adjustable precision pipetters, preferably with disposable plastic tips
- Tubes appropriate for holding cells during induction of ROS/RNS (for suspension cells only)
- Adjustable speed centrifuge with swinging buckets (for suspension cultures)
- Glass microscope slides
- Glass cover slips
- Deionized water
- Anhydrous DMF (100%)

IV. Safety Warnings and Precautions

- This product is for research use only and is not intended for diagnostic purposes.
- The NO Detection Reagent (Red) and ROS Inducer (Pyocyanin) contain DMF which is readily absorbed through the skin. It is harmful if ingested or absorbed through the skin and may cause irritation to the eyes. Observe appropriate precautions when handling.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly.
- 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 blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.
- To avoid photobleaching, perform all manipulations in low light environments or protected from light by other means.

V. Methods and Procedures

NOTE: *Allow all reagents to warm to room temperature before starting with the procedures. Upon thawing of solutions, gently hand-mix or vortex the reagents prior to use to ensure a homogenous solution. Briefly centrifuge the vials at the time of first use, as well as for all subsequent uses, to gather the contents at the bottom of the tube.*

A. REAGENT PREPARATIONS

Reconstitution or dilution of any and all reagents in DMSO should be avoided, as this solvent inhibits hydroxyl radical generation in cells.

1. Dilution of NO Detection Reagent

Dilute the NO Detection Reagent (Red) 1:400 prior to using it in the assay by adding 2.5 µL of the reagent per 1 mL of **pre-warmed** cell culture medium.

2. Positive Control

The NO Inducer (L-Arginine) is supplied at a stock concentration of 100 mM in deionized water. A final concentration of 1mM is recommended. However, the optimal final concentration is cell-dependent and should be determined experimentally for each cell line being tested. NO induction generally occurs within 15-20 minutes upon treatment with NO Inducer (L-Arginine) and may drastically decrease or disappear after that time. Plan accordingly.

3. Negative Control

The NO Scavenger (c-PTIO) is supplied lyophilized and should be reconstituted in 100 µL DMF to produce a stock concentration of 4 mM. A final concentration of 20 µM - 80 µM is recommended. However, the optimal final concentration is cell-dependent and should be determined experimentally for each cell line being tested.

Note: *Adherent cells pretreated with c-PTIO may become weakly adherent and/or detach from the cell culture substratum.*

Endogenous fluorescence of untreated cells should be determined in advance or per assay.

4. 1X Wash Buffer

Allow the 10X Wash Buffer to warm to room temperature. Make sure that the reagent is free of any crystallization before dilution. Prepare enough 1X Wash Buffer for the number of samples to be washed by diluting each milliliter (mL) of the 10X Wash Buffer with 9 mL of deionized water.

B. CELL PREPARATIONS

Cells should be maintained via standard tissue culture practices. Always make sure that cells are healthy and in the log phase of growth before using them for the experiment.

C. FLUORESCENCE/CONFOCAL MICROSCOPY (ADHERENT CELLS)

1. The day before the experiment, seed the cells directly onto glass slides or polystyrene tissue culture plates to ensure ~50-70% confluency on the day of the experiment.

   Important: *Cells should be healthy and not overcrowded since results of the experiments will depend significantly on the cells' condition.*

2. Load the cells with the pre-diluted **NO Detection Reagent (Red)** (see step A-1, page 3) using a volume sufficient to cover the cell monolayer and incubate under normal tissue culture conditions for 2 hours.

3. Carefully remove the NO Detection Mix from the glass slides by gently tapping them against layers of paper towel, or from tissue culture plates. ***Optional****: Cells may be washed with the 1X Wash Buffer.*

4. Treat the cells with an experimental test agent. Separate positive control samples should be treated with the NO Inducer (L-Arginine). Negative Control samples should be established by treatment with the NO Scavenger (c-PTIO).

   Note: *Cells should be treated with the NO Scavenger 15-30 minutes prior to induction.*

   All treatments should be performed under normal tissue culture conditions. It is recommended to perform a pretreatment by adding the NO Scavenger to the aliquots of pre-diluted NO Detection Reagent (Red) for the last 15-30 minutes of the reagent loading. Treatment with an experimental test agent or NO inducers included with the kit should be performed in the cell culture media without dyes.

5. Carefully wash cells twice with 1X Wash Buffer in a volume sufficient to cover the cell monolayer.

   Note*: Adherent cells pre-treated with NO Scavenger (c-PTIO) may become loose and/or detach from the cell culture substratum.*

6. Immediately overlay the cells with a cover slip and observe them under a fluorescence/confocal microscope using a filter set that is compatible with Cyanine 5 (650/670nm). Make sure prepared samples are protected from drying. Dried out cells may present different fluorescence patterns.

D. FLUORESCENCE/CONFOCAL MICROSCOPY (SUSPENSION CELLS)

1. Cells should be cultured to a density not to exceed 1 x 10^6 cells/mL. Make sure that cells are in the log phase of growth before starting an experiment.

   Important: *Cells should be healthy and not overcrowded since results of the experiments will depend significantly on the cells' overall condition. A sufficient volume of cells should be centrifuged at 400 x g for 5 minutes, yielding a working cell count of 1 x 10^5 cells/sample.*

2. Resuspend the cell pellet in 200 µL of pre-diluted **NO Detection Reagent** (see step A-1, page 3) and incubate under normal tissue culture conditions for 2 hours with periodic shaking.

3. Centrifuge the cells at 400 x *g* for 5 minutes to remove the NO Detection Reagent. ***Optional****: Resuspend the cells in 5 mL 1X Wash Buffer,* centrifuge them at 400 x *g* for 5 minutes and remove the supernatant.

4. Treat the cells with an experimental test agent. Separate positive control samples should be treated with the NO Inducer (L-Arginine). Negative Control samples should be established by treatment with the NO Scavenger (c-PTIO).

   Note: *Cells should be treated with the NO Scavenger 15-30 minutes prior to induction.*

   All treatments should be performed under normal tissue culture conditions. It is recommended to perform a pretreatment by adding the NO Scavenger Inhibitor to the aliquots of pre-diluted NO Detection Reagent for the last 15-30 minutes of the reagent loading. Treatment with an experimental test agent or the NO inducer included with the kit should be performed in the cell culture media without dyes.

5. Centrifuge the cells at 400 x *g* for 5 minutes.

6. Resuspend the cells in 5 mL of 1X Wash Buffer, centrifuge them at 400 x *g* for 5 minutes and remove the supernatant.

7. Resuspend the cells in 100 µL of 1X Wash Buffer and apply a 20 µL aliquot of the cell suspension, sufficient for 2 x 10^4 cells, onto a microscope slide. Immediately overlay the cells with a cover slip and analyze immediately via fluorescence microscopy. Make sure that prepared samples are protected from drying. Dried out cells may present different fluorescence patterns. NO detection requires a filter set compatible with Cyanine 5 (650/670nm).

VI. Appendices

A. FILTER SET SELECTION

For fluorescence microscopy, careful consideration must be paid to the selection of filters. Dichroic filters should be selected in which the "cut-off" frequency is optimally mid-way between the two emission bands that are desired (one reflected, the other transmitted). However, it is important to realize that dichroic filters have a somewhat limited reflectance range, *i.e.,* a 600 nm short-pass dichroic filter may actually reflect light <500 nm. When selecting filters, it is critical to discuss with the filter or microscope manufacturer exactly what wavelength specifications are required for both the transmitted and the reflected light.

In addition, filters should be obtained that have the highest possible transmission efficiency (typically requiring anti-reflection coating). Each optic that an emission beam must traverse will remove some fraction of the desired light. The difference between 80% transmission and 95% transmission for each detector may result in a greater than three-fold difference in the amount of light available to the detector.

B. SETTING UP OPTIMAL EXPOSURE TIME FOR DETECTION OF THE DYE

Optimal exposure times should be established experimentally for each dye used in the experiment. Both negative and positive controls should be utilized. Start with the negative control (untreated stained cells) and set up the exposure time so the fluorescent background is negligible. Then switch to a positive control (arginine or pyocyanin treated cells) and adjust the exposure time to record a bright fluorescent image. Avoid saturation of the signal (very bright spots on the image). If saturation of the signal occurs, decrease the exposure time. It is recommended to acquire 5-6 single color images for each sample.

C. ANTICIPATED RESULTS

1. In the presence of NO, the NO Detection Reagent (Red) will demonstrate a red punctuate cytoplasmic staining pattern. Keep in mind that fluorescence of the NO Detection Reagent (Red) is not very bright.
2. Nitric oxide (NO) positive control samples, induced with NO Inducer (L-Arginine), should exhibit red fluorescence, with punctuate cytoplasmic staining pattern.
3. Cells incubated with the NO Scavenger (c-PTIO), post-induced with NO Inducer (L-Arginine) should not demonstrate red fluorescence.
4. Untreated samples should present only low autofluorescent background signal in any channel.

VII. References

1. Tarpey, M. and Fridovich, I. Circ Res. **89** (2001), 224-236.
2. Batandier ,C., *et al.* J Cell Mol Med. **6** (2002), 175-187.
3. Gomes, A., *et al*. J Biochem Biophys Meth. **65** (2005), 45-80.
4. Wardman, P. Free Rad Biol Med. **43** (2007), 995-1022.

VII. Troubleshooting Guide

Problem	Potential Cause	Suggestion
Low or no fluorescent signal in positive control	Dead or stressed (overcrowded) cells	Prepare fresh cell culture for the experiments. Make sure that the cells are in the log growth phase.
	Band pass filters are too narrow or not optimal for fluorescent probes.	Multiple band pass filters sets provide less light than single band pass ones. Use correct filter set(s). Check Methods and Procedures section of this manual and Appendix A for recommendations. Use of a wide band pass or a long pass filters is recommended.
	Insufficient fluorescent dye concentration	Follow the procedures provided in this manual.
	Insufficient inducer concentration	Determine an appropriate concentration of inducer for the cell line(s) used in the study.
	Species of interest may react with each other, thus attenuating the expected signal.	Check signaling pathways and all the components present in the cellular environment.
	Inappropriate time point of the detection	Make sure that time of detection is optimized and the samples are prepared immediately. Red signal may disappear over time because the NO oxidized fluorescent probe can undergo further redox changes in the cellular physiological environment.

Problem	Potential Cause	Suggestion
High fluorescent background	Stressed (overcrowded) cells	Prepare new cell culture for the experiment. Make sure that the cells are in the log growth phase.
	Band pass filters are too narrow or not optimal for fluorescent probes.	Use correct filter for the fluorophore. Check Methods and Procedures section of this manual and Appendix A for the recommendations. Minimal spectral overlap should occur with the selected set of filters.
	Wash step is necessary.	Follow the procedures provided in this manual, making optional wash steps mandatory.
	Inappropriate time point for detection	Make sure that time of detection is optimized and the samples are prepared immediately.
	Inappropriate cell conditions	Make sure that you have viable cells at the beginning of the experiment, and that the inducer treatment does not kill the cells during the time frame of the experiment .
No decrease of the fluorescent signal after using a specific inhibitor.	Inappropriate inhibitor concentration (too low or too high)	Very low doses of inhibitor may not affect NO production by inducer. Alternatively, very high doses of the inhibitors may cause stress itself and generate fluorescent signal. Optimize the concentration of the inhibitor and pretreatment time for each particular cell line.
	Inappropriate time point for detection	When cells are kept too long with the inhibitors or at very high inducer concentrations, after a certain time, the inhibitor becomes insufficient. Make sure that time of detection is optimized.
	Inappropriate filter set on the microscope	Use correct filter. Check Methods and Procedures section of this manual and Appendix A for the recommendations.

Enzo Life Sciences

www.enzolifesciences.com

North/South America
ENZO LIFE SCIENCES INTERNATIONAL, INC.
5120 Butler Pike
Plymouth Meeting, PA 19462-1202
USA
Tel. 1-800-942-0430 / (610) 941-0430
Fax (610) 941-9252
info-usa@enzolifesciences.com

Switzerland & Rest of Europe
ENZO LIFE SCIENCES AG
Industriestrasse 17, Postfach
CH-4415 Lausen / Switzerland
Tel. + 41/0 61 926 89 89
Fax + 41/0 61 926 89 79
info-ch@enzolifesciences.com

Benelux
ENZO LIFE SCIENCES BVBA
Melkerijweg 3
BE-2240 Zandhoven / Belgium
Tel. +32/0 3 466 04 20
Fax +32/0 3 466 04 29
info-be@enzolifesciences.com

Germany
ENZO LIFE SCIENCES GmbH
Marie-Curie-Strasse 8
DE-79539 Lörrach / Germany
Tel. +49/0 7621 5500 526
Fax +49/0 7621 5500 527
info-de@enzolifesciences.com

UK & Ireland
ENZO LIFE SCIENCES (UK) LTD.
Palatine House
Matford Court
Exeter EX2 8NL / UK
Tel. 0845 601 1488 (UK customers)
Tel. +44/0 1392 825900 (overseas)
Fax +44/0 1392 825910
info-uk@enzolifesciences.com

For Local Distributors please visit our Website.