Nitric Oxide Synthase Assay
Catalog #9155

1. INTRODUCTION

Nitric oxide synthases are a family of enzymes capable of catalyzing the production of nitric oxide (NO). NO is an important molecule that is involved in regulating a variety of cellular processes such as angiogenesis, peristalsis, and the immune response to invading pathogens[1,2]. Reactive nitrogen species (RNS) are a family of antimicrobial molecules derived from NO and superoxide through the enzymatic activities of nitric oxide synthase (NOS)[2].

ICT's Nitric Oxide Synthase Assay provides a good screening option for assessing the potency of nitrosative stress inhibitor and activator reagents, and will help to determine how oxidative and nitrosative stress modulates varied intracellular pathways. This kit assesses the overall intracellular levels of free nitric oxide and NOS using a Diaminofluorescein-2 Diacetate (DAF-2DA) dye[3,4].

The kit provides all the essential reagents and an easy to follow protocol to assess changes in intracellular NO and NOS levels using flow cytometry, fluorescence plate reader, and fluorescence microscopy. The DAF-2DA dye quickly penetrates membrane structures and accumulates within the cell. Once inside the cell the diacetate groups on the DAF-2DA reagent are hydrolyzed by cytosolic esterases, allowing for the release and sequestration of DAF-2 inside the cell. Production of nitric oxide converts the non-fluorescent DAF-2 dye to its fluorescent triaole derivative, DAF-2T[3-7].

Nitric Oxide Synthase Assay requires minimal procedural steps and hands-on time to complete. Samples are stained for 1 hour with DAF-2DA dye. Cells are washed to remove excess dye, and then treated experimentally. Since the unbound reagent is non-fluorescent, subsequent wash steps are not required, thus simplifying the assay procedure. Following treatment, cells are ready for analysis by flow cytometry.

Each kit will enable the assessment of up to 50 (0.5 mL) or 100 (0.25 mL) samples. For microscopy usage, Hoechst 33342 is included with the kit to concurrently label nuclei after labeling with DAF-2DA. DAF-2DA optimally excites at 488 nm, and has a peak emission at 515 nm. Hoechst 33342 can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm.

2. KIT CONTENTS

- 1 vial of Diaminofluorescein-2 Diacetate (56 µL), #6697
- 1 vial of Hoechst 33342, 200 µg/mL (1 mL), #639
- 1 bottle of 10X Assay Buffer (60 mL), #685

3. STORAGE

Store the unopened kit and each unopened component at 2-8°C until the expiration date. Protect DAF-2DA dye from light.

4. SAFETY DATA SHEETS (SDS)

Safety data sheets are available online at www.immunochemistry.com or by calling 1-800-824-8540.

5. RECOMMENDED MATERIALS

- 15 mL polypropylene centrifuge tubes (1/sample)
- DiH_2O (540 mL per bottle to dilute 10X Assay Buffer)
- FACS tubes
- Cultured cells treated with the experimental conditions ready to be labeled
- Reagents to induce NOS activity, such as DEA NONOate (e.g. Enzo #ALX-430-034).
- Hemocytometer
- Centrifuge at <200 g
- Cell culture supplies, such as media and incubator

6. DETECTION EQUIPMENT

This assay can be analyzed with a:

- Flow cytometer
- Fluorescence microscope
- Fluorescence plate reader

Use filter pairing that best approximate these settings:

- DAF-2DA optimally excites at 488 nm, and has a peak emission at 515 nm. Hoechst optimally excites at 365 nm and has a peak emission at 480 nm.

7. PREPARATION OF DAF-2DA STAINING SOLUTION

DAF-2DA dye is supplied in a vial containing 56 µL at a concentration of 0.125 mg/mL. Immediately prior to staining, dilute the dye by adding 56 µL of the supplied stock solution to 444 µL of water.

The dye is then ready to use. To stain 50 x 0.5 mL samples, add 10 µL of diluted dye to 490 µL of sample. To stain 100 x 0.25 mL samples, add 5 µL of diluted dye to 245 µL of sample.

If not using all of the supplied DAF-2DA, adjust dilution volume accordingly. Undiluted (in DMSO) DAF-2DA may be stored at 2-8°C for up to 1 year.

Manufactured by Daiichi Pure Chemicals Co. Ltd. Not for sale in Japan

8. PREPARATION OF 1X ASSAY BUFFER

1. Allow 10X Assay Buffer to come to room temperature. A precipitate may have formed during during storage at 2-8°C. Make sure to allow sufficient time for the precipitate to go back into solution.
2. Dilute 1:10. For example, add 60 mL of 10X Assay Buffer to 540 mL of deionized or distilled water.
3. Filter sterilize 1X Assay Buffer prior to use. May store sterile 1X Assay Buffer for up to 1 month at ≤ 2-8°C. Buffer does not contain presevatives. Avoid contamination. Do not use if buffer appears to be contaminated.

Other isotonic buffers such as Hanks Balanced Salt Solution (HBSS) or Phosphate Buffered Saline (PBS) may be used instead of the provided Assay Buffer. Avoid buffers containing serum, BSA, or phenol red. Alternate buffers should be evaluated by the end user.

9. PREPARATION OF HOECHST 33342

Hoechst 33342 is a cell-permeant nuclear stain that emits blue fluorescence when bound to double stranded DNA. It is used to stain the nuclei of living or fixed cells, to distinguish condensed pyknotic nuclei in apoptotic cells, and for cell cycle studies.

Hoechst 33342 is provided ready-to-use at 200 µg/mL. Hoechst 33342 can be used with DAF-2DA to label the DNA of live and dead cells. To use, add to samples at 0.5% v/v, and incubate 5 minutes at 37°C. For example, if the cell suspension or overlay medium volume is at 250 µL, add 1.25 µL Hoechst 33342, if medium volume is 500 µL, add 2.5 µL Hoechst 33342.

When bound to DNA, it has a maximum absorbance at 350 nm and a maximum emission at 480 nm. It is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm.

- Hoechst 33342 contains a low concentration of Bis benzimide H 33342 trihydrochloride which is below the threshold for reporting on the safety data sheet (SDS). It is a suspected mutagen at high concentrations. Prolonged skin contact may cause redness and irritation. Because of the small quantity of product, the health hazard is small. See SDS for further information.

10. EXPERIMENTAL PREPARATION

Staining cells with DAF-2DA can be completed within 30-60 minutes. However, DAF-2DA is used with living cells, which require periodic maintenance and cultivation several days in advance. In addition, once the proper number of cells has been pre-loaded with DAF-2DA, additional time must be allotted for the experimental treatment, which may vary. The recommended sample size is 0.25-0.5 mL of cells at 5 x 10^5 cells/mL.

After staining with DAF-2DA, create cell populations, such as:

a. Cells that were exposed to the experimental treatment.

b. A negative control population of cells that received a placebo treatment.

Culture cells to a density optimal for the specific experimental protocol. Cell density should not exceed 10^6 cells/mL. Cells cultivated in excess of this concentration may begin to naturally enter apoptosis. An initial experiment may be necessary to determine how much DAF-2DA to use.

- **DAF-2DA is not compatible with fixed samples. Organic solvents and formalin will denature enzymes and give false readings.**

11. EXPERIMENTAL SAMPLES AND CONTROLS

Create experimental samples and control cell populations:

a. Treated experimental population(s): cells exposed to the experimental condition(s).

b. Positive control for NO production or NOS activity: for example, cells exposed to DEA NONOate for 1 hour.

c. Negative control for NO production or NOS activity: untreated or mock-treated cells.

12. CELL STAINING PROTOCOL

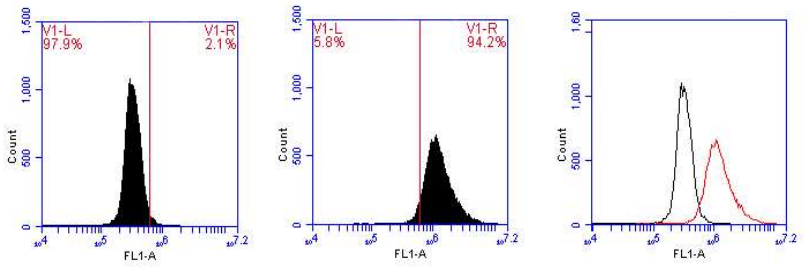
Prepare experimental and control cell populations. Ideally, the cell concentration should be 3-5 x 10^5 cells/mL. The concentration should not exceed 10^6 cells/mL, as cells cultivated in excess of this concentration may begin to naturally enter apoptosis. Cells may need to be concentrated to 2-5 x 10^6 cells/mL after staining as microscopy and fluorescence plate reader analysis methods (Sections 14 and 15, respectively) require high cell concentrations. Flow cytometry (Section 13) can analyze samples at 3-5 x 10^5 cells/mL.

When using a fluorescence plate reader to analyze adherent cells, culture cells on tissue culture plates with clear bottoms and black walls. Culture cells to approximately 90% confluency.

1. If the samples are cultured in medium containing serum, BSA, or phenol red, wash with 1X Assay Buffer to remove prior to staining. PBS or Hanks Balanced Salt Solution (HBSS) may be used as an alternative wash buffer.
2. Remove wash buffer and replace with 1X Assay Buffer or an alternative buffer that does not contain serum, BSA, or phenol red.
3. Stain samples (0.5 mL or 0.25 mL) with diluted DAF-2DA by adding 10 µL of diluted dye to 490 µL or add 5 µL of diluted dye to 245 µL of sample, respectively.

FIGURE 1: ANALYSIS VIA FLOW CYTOMETRY

Jurkat cells were stained with ICT's DAF-2DA dye (Kit #9155) for 1 hour, washed, and then mock-treated with DMSO to create a negative control (left histogram) or 1 mM DEA NONOate, a nitric oxide donor (middle histogram), for 30 minutes at 37°C. Cells were read on the FL1 channel of an Accuri C6 flow cytometer. The median fluorescence intensity (MFI) of stained cells in the negative control was 324,817 in FL1-A (left: Negative), whereas the treated population had a value of 1,190,365 (middle: Positive), which is a increase of more than 3.5-fold. The effect of DEA NONOate on intracellular NOS activity is easily visible when the samples are overlaid in a single plot (right, black: Negative; right, red: Positive). Data courtesy of Dr. Kristi Strandberg, ICT, 227:76.



Negative, treated with DMSO

Positive, Treated with DEA NONOate

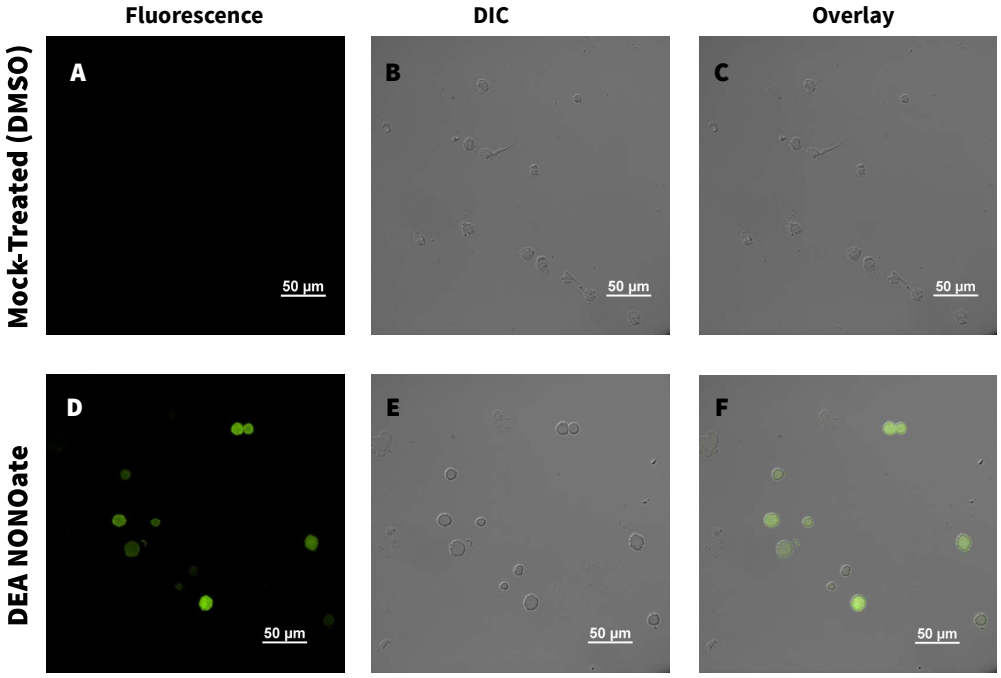
Overlay

FIGURE 2: ANALYSIS VIA FLUORESCENCE MICROSCOPE

Jurkat suspension cells were stained with DAF-2DA dye for 1 hour, and then washed. Cells were then treated with DMSO as a negative control (Panels A-C) or 1 mM DEA NONOate (Panels D-F), a nitric oxide donor, for 30 minutes at 37°C.

Panels A and D reveals green fluorescence-stained cells. Panels B and E show a corresponding differential interference contrast (DIC) image, which reveals cell morphology. Panels C and F show an overlay of the fluorescence and DIC images. Cells treated with DEA NONOate showed an increased level of green fluorescence relative to the untreated cells.

Microscope images were obtained using a Nikon Eclipse 90i microscope with a Hamamatsu Flash 4.0 camera. Data courtesy of Dr. Kristi Strandberg (ICT 228:57-58).



4. Incubate the cells with DAF-2DA dye protected from light. The incubation period may range from 30 to 60 minutes and should be optimized for each cell line and experimental condition.

• Reducing the incubation temperature will lessen the sub-cellular compartmentalization of the dye.

5. Wash the samples at least 1 time with equal or greater volume of 1X Assay Buffer to remove excess dye.
6. Remove wash buffer and replace with 0.5 mL or 0.25 mL (depending on starting sample size) of 1X Assay buffer or buffer of choice (avoiding buffers containing serum, BSA, or phenol red).
7. Expose cells to the experimental or control conditions. Set aside two populations to create positive and negative controls (Section 11).
   a. To analyze using a flow cytometer, refer to Section 13.
   b. To analyze using a fluorescence microscope, refer to Section 14.
   c. To analyze using a fluorescence plate reader, refer to Section 15.

• Additional wash steps are not required, as any DAF-2 that has not been converted to its triazole derivative (DAF-2T) will be non-fluorescent.

13. FLOW CYTOMETRY ANALYSIS

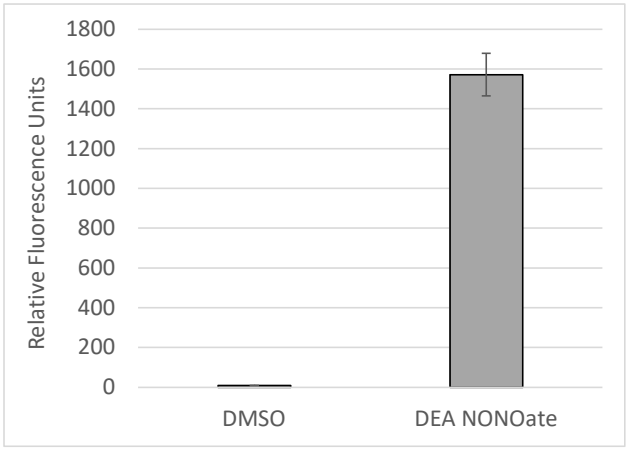
Follow Section 12 (Steps 1-7). Sample flow cytometry data is shown in Figure 1.

8. Samples are ready for analysis. No further processing is required. To analyze the samples, measure green fluorescence using an excitation at 488 nm and emission at 515 nm (FL-1 channel).

FIGURE 3: ANALYSIS VIA FLUORESCENCE PLATE READER

Jurkat suspension cells were stained with DAF-2DA dye for 1 hour, and then washed. Cells were then treated with DMSO as a negative control or 1 mM DEA NONOate, a nitric oxide donor, for 30 minutes at 37°C. Cells were analyzed using a fluorescence plate reader using an excitation at 488 nm and emission at 515 nm.

Data courtesy of Dr. Strandberg, ICT, 227:39-42.



14. MICROSCOPY ANALYSIS

Follow Section 12 (Steps 1-7). Sample microscopy data is shown in Figure 2.

8. Optional Hoechst 33342 staining: If dual staining with Hoechst, add Hoechst at 0.5% v/v and incubate for 5 minutes at 37°C. For example, add 2.5 µL of Hoechst to a 500 µL suspension cell sample, or add 1.25 µL of Hoechst to a 250 µL suspension cell sample.
9. When ready to view cells, place 1 drop of cell suspension onto a microscope slide and cover with a coverslip.
10. Observe cells under a fluorescence microscope using excitation at 470-490 plus a >520 nm long pass emission filter. Live cells fluoresce green. Observe Hoechst staining using a UV-filter with excitation at 365 nm and emission at 480 nm.

15. FLUORESCENCE PLATE READER ANALYSIS

Follow Section 12 (Steps 1-7). Sample fluorescence plate reader results are shown in Figure 3.

8. Determine the concentration and compare the cell density of each sample. The negative control/mock-treated population may have more cells than the positive control/NOS activiated population. Adjust the volume of the cell suspensions to equalize the cell density. When ready to read, cells should be between 2-5 x 10^6 cells/mL.
9. Pipette 100 µL cell suspension per well into a black-opaque-well microtiter plate. It is recommended that each sample be tested in duplicate (at a minimum).

• Do NOT use clear plates. Avoid bubbles.

10. Perform an endpoint read. If possible, set the excitation wavelength to 488 nm and the emission wavelength to 515 nm.

16. REFERENCES AND CITATIONS

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