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# ab211084
# Nitric Oxide Synthase Activity Assay Kit (Fluorometric)

For the rapid, sensitive, and accurate measurement of Nitric Oxide Synthase (NOS) activity in cell or tissue lysates.

This product is for research use only and is not intended for diagnostic use.

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# 1. Overview

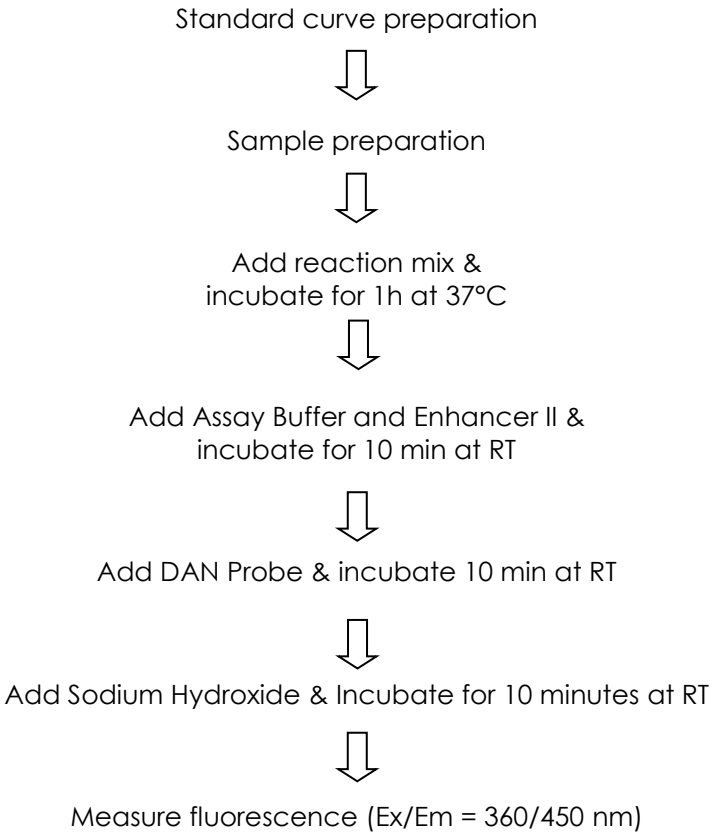
Nitric Oxide Synthase (NOS) Activity Assay Kit (Fluorometric) (ab211084) provides an accurate and convenient method to assay NOS activity in cell and tissue lysates. In this assay, nitric oxide generated by NOS undergoes a series of reactions and reacts with the fluorescent probe to generate a stable signal at Ex/Em = 360/450 nm, which is directly proportional to NOS activity.

The assay is simple, sensitive and high-throughput adaptable and can detect as low as 0.75 µU of NOS activity.

Nitric oxide synthases (EC 1.14.13.39) (NOS) are a family of enzymes that catalyze the production of nitric oxide (NO) from L-arginine. Nitric oxide (NO) plays an important role in neurotransmission, vascular regulation, immune response and apoptosis. In presence of NADPH, FAD, FMN, (6R)-5,6,7,8-tetrahydrobiopterin, calmodulin and heme, NOS catalyzes a five-electron oxidation of the guanidino nitrogen of L-arginine with molecular oxygen to generate NO and L-citrulline.

There are three isoforms of NOS: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). nNOS accounts for the production of NO in central nervous system, where NO participates in cell communication and information storage. eNOS produces NO in blood vessels and is involved in regulation of vascular function. In contrast to other isoforms, iNOS is expressed de novo under oxidative stress conditions and produces large amounts of NO as a part of body's defense mechanism.

## 2. Protocol Summary



## 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 pipette 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

**All components in this kit are shipped on blue ice and are suitable for storage at -80°C, unless reconstituted. Upon receipt, immediately store kit at -80°C in the dark. Individual components may be stored at alternative temperatures as show in the table below. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.** 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.

Aliquot components in working volumes before storing at the recommended temperature.

**Δ Note:** Reconstituted components are stable for 2 months.

## 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 temperature (before prep) | Storage temperature (after prep) |
|---|---|---|---|
| NOS Assay Buffer | 25 mL | -80°C | 4°C or -20°C |
| NOS Dilution Buffer | 1.5 mL | -80°C | 4°C or -20°C |
| NOS Substrate | 0.5 mL | -80°C | - 20°C |
| Cofactor I | 1 vial | -80°C or -20°C | -20°C |
| 25X NOS Cofactor 2 | 100 µL | -80°C | -20°C |
| Nitrate Reductase II | 1 vial | -80°C | -20°C |
| NOS Positive Control | 4 µL | -80°C | -80°C |
| Enhancer II | 1 vial | -80°C or -20°C | -20°C |
| Nitrite Standard | 1 vial | -80°C or -20°C | 4°C |
| DAN Probe | 1 mL | -80°C or -20°C | 4°C |
| Sodium Hydroxide | 1 mL | -80°C or -20°C | 4°C |

PLEASE NOTE: Cofactor I was previously labelled as NOS Cofactor I and NOS Cofactor 1 (1 µmole). The composition has not changed.

## 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 fluorescence at Ex/Em = 360/450 nm
- Deionized water or other type of double distilled water (dd$H_2O$)
- PBS
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom, preferably white
- Dounce homogenizer (if using tissue)
- BCA protein assay kit (reducing agent compatible): we recommend using BCA protein assay kit reducing agent compatible (microplate) (ab207003)
- Protease inhibitor cocktail: we recommend Protease Inhibitor Cocktail (ab65621)

## 8. Technical Hints

- **This kit is sold based on number of tests. A "test" simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Samples which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

## 9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

**9.1 NOS Assay Buffer:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C or -20°C.

**9.2 NOS Dilution Buffer:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C or -20°C.

**9.3 NOS Substrate (500 µL):**

Ready to use as supplied. Keep on ice while in use. Aliquot substrate so that you have enough to perform the desired number of assays. Avoid repeated freeze/thaw. Store at -20°C.

**9.4 Cofactor I:**

Reconstitute Cofactor I in 110 µL of $ddH_2O$ to make 10 mM stock solution. Aliquot so that you have enough to perform the desired number of assays. Store at -20°C. Limit freeze/thaw to 1 time.

Just before use, make **1mM Working Solution Cofactor I** by diluting the 10 mM stock in $ddH_2O$. Make as much working solution as needed. Keep on ice while in use. Working solution can be stored at 4°C for 6 – 8 hours.

**9.5 25X NOS Cofactor 2:**

Just before use, make **1X Working Solution NOS Cofactor 2** by diluting stock in $ddH_2O$. Make as much working solution as needed. Keep on ice while in use.

Aliquot stock solution(25X) so that you have enough volume to perform the desired number of assays. Store at -20°C. Avoid repeated freeze/thaw.

**9.6 Nitrate Reductase II:**

Reconstitute Nitrate Reductase II in 1.1 mL of NOS Assay Buffer. Keep on ice while in use. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Store at -20°C. Avoid repeated freeze/thaw.

**9.7 NOS Positive Control:**

Keep the solution on ice at all times while in use since the enzyme loses activity at higher temperature. Immediately prior use, dilute NOS enzyme 1:20 in NOS Dilution Buffer.

Aliquot enzyme so that you have enough to perform the desired number of assays. Store at -80°C. Limit freeze/thaw to 1 time.

**9.8 Enhancer II:**

Reconstitute Enhancer II in 1.2 mL of NOS Assay Buffer. Aliquot Enhancer II so that you have enough to perform the desired number of assays. Keep on ice while in use. Store at -20°C.

**9.9 Nitrite Standard:**

Reconstitute Nitrite Standard in 1 mL of NOS Assay Buffer to generate a 10 mM standard. Vortex and mix well. Do not freeze. Store at 4°C. Reconstituted standard is stable for 4 months when stored at 4°C.

**9.10 DAN Probe:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

**9.11 Sodium Hydroxide:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

## 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.

**10.1** Prepare a 50 µM Nitrite working standard solution by adding 5 µL of 10 mM Nitrite Standard to 995 µL of Assay Buffer.

**10.2** Using 50 µM Nitrite working standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

| Standard# | Nitrite 50 µM standard (µL) | Assay Buffer (µL) | Final volume standard in well (µL) | End amount Nitrite in well (pmol/well) |
|---|---|---|---|---|
| **1** | 0 | 120 | 40 | 0 |
| **2** | 12 | 108 | 40 | 200 |
| **3** | 24 | 96 | 40 | 400 |
| **4** | 36 | 84 | 40 | 600 |
| **5** | 48 | 72 | 40 | 800 |
| **6** | 60 | 60 | 40 | 1000 |

Each dilution has enough amount of standard to set up duplicate readings (2 x 40 µL).

# 11. Sample Preparation

**General sample information:**

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze your samples in liquid nitrogen upon extraction and store them immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.
- Add protease inhibitor cocktail to the assay buffer immediately prior to use.

## 11.1 Cell lysates:

11.1.1 Harvest the required amount of cells necessary for each assay (initial recommendation 2-5 x $10^6$ cells).
11.1.2 Wash cells with cold PBS.
11.1.3 Resuspend cells in 100-200 µL of cold NOS Assay Buffer (containing protease inhibitor cocktail) on ice.
11.1.4 Homogenize cells quickly by pipetting up and down a few times.
11.1.5 Centrifuge sample for 10 minutes at 4°C at 10,000 x *g* using a cold microcentrifuge to remove any insoluble material.
11.1.6 Collect supernatant and transfer to a new pre-chilled tube.
11.1.7 Keep on ice.
11.1.8 Measure the amount of protein in the cell lysate using a BCA Protein Assay kit.
11.1.9 Proceed to assay NOS activity immediately.

## 11.2 Tissue lysates:

11.2.1 Harvest the amount of fresh or frozen tissue necessary for each assay (initial recommendation ~ 100 mg).
11.2.2 Wash tissue in cold PBS.
11.2.3 Add 200 µL of cold NOS Assay Buffer (containing protease inhibitor cocktail) to the tissue.
11.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.

11.2.5 Centrifuge sample for 10 minutes at 4°C at 10,000 x *g* using a cold microcentrifuge to remove any insoluble material.
11.2.6 Collect supernatant and transfer to a new pre-chilled tube.
11.2.7 Keep on ice.
11.2.8 Measure the amount of protein in the lysate or purified enzyme using a BCA Protein Assay kit.
11.2.9 Proceed to assay NOS activity immediately.

**Δ Note**: We suggest using different volumes of sample to ensure readings are within the standard curve range.

## 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.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

**Δ Note**: If you suspect your samples contain substances that can generate background, set up Sample Background Controls to correct for background noise.

### 12.1 Plate Loading:

- Standard wells = 40 µL standard dilutions.
- Positive control = 5 – 10 µL diluted positive control (1:20) (adjust volume to 40 µL/well with NOS Assay Buffer).
- Sample wells = 20 - 40 µL (200 – 400 µg protein) sample into each well (adjust volume to 40 µL/well with NOS Assay Buffer).
- (Optional) Sample Background Control wells = 20 - 40 µL (200 – 400 µg protein) sample into each well. Adjust final volume to 200 µL/well with NOS Assay Buffer.

### 12.2 NOS Reaction Mix:

12.2.1 Prepare 18 µL of Reaction Mix for each reaction. Mix enough reagents for the number of assays to be performed. Prepare a master mix to ensure consistency.

| Component | Reaction Mix (µL) |
|---|---|
| Diluted Cofactor I | 5 |
| NOS Cofactor 2 (1X) | 3 |
| NOS Substrate | 5 |
| Nitrate Reductase II | 5 |

12.2.2 Add 18 µL of Reaction Mix into standard, positive control and sample well. DO NOT add reaction mix to background control wells.

12.2.3 Mix well by pipetting up and down.

12.2.4 Incubate at 37°C for 1 hour.

12.2.5 Add 122 µL of NOS Assay Buffer to standard, positive control and sample well. DO NOT add reaction mix to background control wells. Mix well by pipetting up and down.
12.2.6 Add 5 µL of Enhancer II to standard, positive control and sample well. DO NOT add Enhancer II to background control wells.
12.2.7 Mix well by pipetting up and down.
12.2.8 Incubate at room temperature for 10 minutes.

**12.3 Measurement:**

12.3.1 Add 10 µL of DAN Probe to standard, positive control and sample well. DO NOT add DAN Probe to background control wells. Mix well by pipetting up and down.
12.3.2 Incubate at room temperature for 10 minutes.
12.3.3 Add 5 µL of Sodium Hydroxide to standard, positive control and sample well. DO NOT add Sodium Hydroxide to background control well.
12.3.4 Mix well by pipetting up and down.
12.3.5 Incubate at room temperature for 10 minutes.
12.3.6 Measure fluorescence (for all wells) on a microplate reader at Ex/Em = 360/450 nm.

## 13. Calculations

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.
- Use only the linear rate for calculation.

**13.1** Subtract the mean fluorescence value of the blank (Standard #1) from all standard and sample readings. This is the corrected fluorescence.

**13.2** Standard curve calculation:

13.2.1 Average the duplicate reading for each standard.

13.2.2 Plot standard curve readings and draw the line of the best fit to construct the standard curve (most plate reader software or Excel can do this step). Calculate the trend line equation based on your standard curve data (use the equation that provides the most accurate fit).

**13.3** Measurement of NOS in the sample:

13.3.1 Subtract the sample background control from sample reading if significant.

13.3.2 Apply variation of fluorescence in the sample (ΔRFU) to the Standard curve to get B pmoles of nitrite generated during the reaction.

13.3.3 Nitrite activity (pmol/min/µg or µU/µg or mU/mg) in the test samples is calculated as:

$$NOS\ Specific\ Activity = \left(\frac{B}{T\ x\ C}\right)$$

Where:

B = Nitrite amount in sample well from Standard Curve (pmol).

T = Reaction time (minutes) (60 minutes).

C = is amount of protein (µg)

Unit definition:

1 Unit NOS activity is the amount of enzyme required to yield 1 µmol of nitric oxide/minute at 37°C under the assay conditions.

## 14. Typical Data

**Typical standard curve** – data provided for **demonstration purposes** only. A new standard curve must be generated for each assay performed.

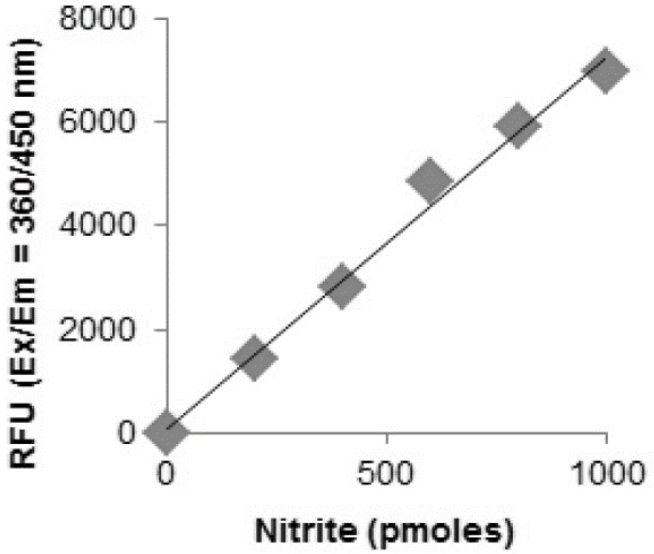



**Figure 1**. Typical standard calibration curve.

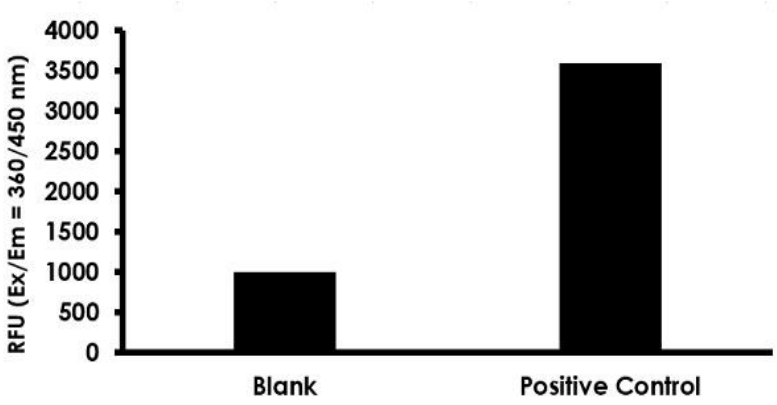



**Figure 2**. Measurement of NOS Positive Control activity (5 µL) compared to blank (Standard #1).

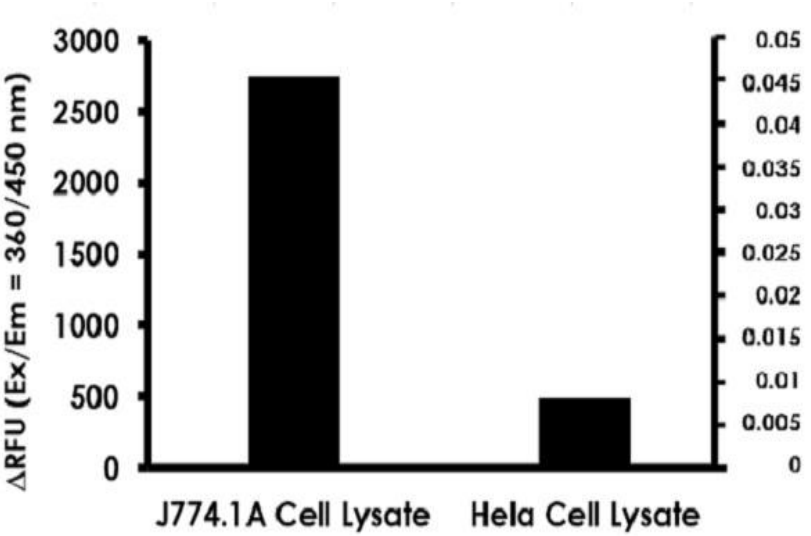



**Figure 3**. Detection of endogenous NOS activity in lysates from J774.1A mouse monocytes (135 µg) and HeLa cells (157 µg).

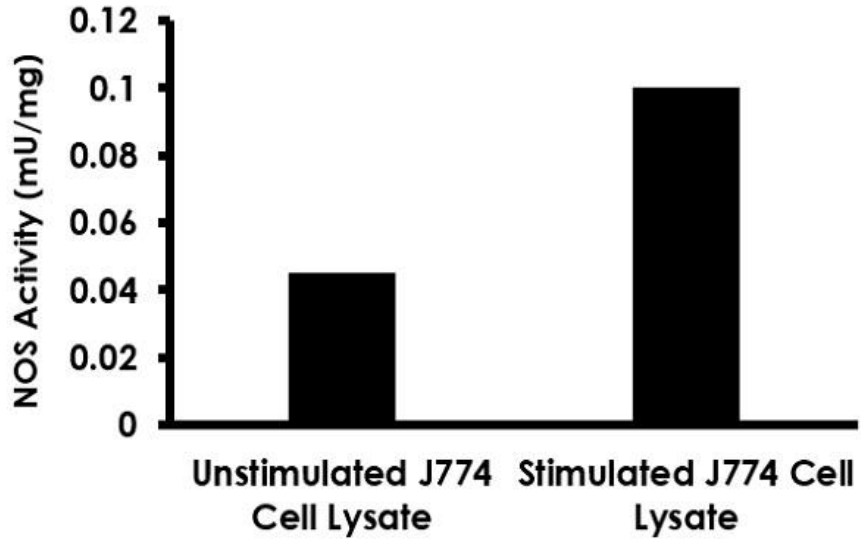



**Figure 4.** Detection of endogenous NOS activity in J774.1A cell lysates. J774 cells were stimulated with 200 ng/mL LPS and 100 ng/mL murine IFN-gamma. Unstimulated control included. Cell lysates were prepared (225 µg) and assayed following the kit protocol.

## 15. Quick Assay Procedure

**Δ Note: this procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.**

- Prepare reagents and aliquot if necessary; get equipment ready.
- Prepare NOS standard dilution [200 – 1000 pmol/well].
- Prepare samples in optimal dilutions to fit standard curve readings.
- Set up plate in duplicate for standard (40 µL), sample (20 - 40 µL) and positive control wells (40 µL).

  Sample background control wells, use 20 – 40 µL sample and adjust to 200 µL with NOS Assay Buffer. Do not add any other reagent to these wells.
- Prepare a master mix for NOS Reaction Mix:

| Component | Reaction Mix (µL) |
|---|---|
| Diluted Cofactor I | 5 |
| NOS Cofactor 2 (1X) | 3 |
| NOS Substrate | 5 |
| Nitrate Reductase II | 5 |

- Add 18 µL Reaction mix to sample, standard and positive control wells.
- Incubate at 37°C for 60 minutes.
- Add 122 µL NOS Assay Buffer and 5 µL Enhancer II to sample, standard and positive control wells.
- Incubate at room temperature for 10 minutes.
- Add 10 µL DAN Probe to sample, standard and positive control wells.
- Incubate at room temperature for 10 minutes.
- Add 5 µL Sodium Hydroxide to sample, standard and positive control wells.
- Incubate at room temperature for 10 minutes.
- Measure fluorescence at Ex/Em = 360/450 nm on a microplate reader.

## 16. Troubleshooting

| Problem | Reason | Solution |
|---|---|---|
| **Assay not working** | Use of ice-cold buffer | Buffers must be at assay temperature |
| | Plate read at incorrect wavelength | Check the wavelength and filter settings of instrument |
| | Use of a different microplate | Colorimetric: clear plates<br>Fluorometric: black wells/clear bottom plates<br>Luminometric: white wells/clear bottom plates |
| **Sample with erratic readings** | Cells/tissue samples not homogenized completely | Use Dounce homogenizer, increase number of strokes |
| | Samples used after multiple free/ thaw cycles | Aliquot and freeze samples if needed to use multiple times |
| | Use of old or inappropriately stored samples | Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use |
| | Presence of interfering substance in the sample | Check protocol for interfering substances |
| **Lower/higher readings in samples and standards** | Improperly thawed components | Thaw all components completely and mix gently before use |
| | Allowing reagents to sit for extended times on ice | Always thaw and prepare fresh reaction mix before use |
| | Incorrect incubation times or temperatures | Verify correct incubation times and temperatures in protocol |

| Problem | Reason | Solution |
|---|---|---|
| **Standard readings do not follow a linear pattern** | Pipetting errors in standard or reaction mix | Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible |
| | Air bubbles formed in well | Pipette gently against the wall of the tubes |
| | Standard stock is at incorrect concentration | Always refer to dilutions described in the protocol |
| **Unanticipated results** | Measured at incorrect wavelength | Check equipment and filter setting |
| | Samples contain interfering substances | Troubleshoot if it interferes with the kit |
| | Sample readings above/ below the linear range | Concentrate/ dilute sample so it is within the linear range |

## 17. Notes

## Technical Support



**For all technical or commercial enquiries please go to:**

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)