

## Measure-iT™ High-Sensitivity Nitrite Assay Kit (M36051)

### Quick Facts

#### Storage upon receipt:

- Store at  $\leq -20^{\circ}\text{C}$
- Protect from light
- Avoid freeze/thaw cycles

**Ex/Em:** 365/450 nm

### Introduction

The Measure-iT™ High-Sensitivity Nitrite Assay Kit provides easy and accurate quantitation of nitrite. The kit supplies concentrated assay reagent, nitrite standard, and developer. Simply dilute the reagent 1:100, load 100  $\mu\text{L}$  into the wells of a microplate, add 1–10  $\mu\text{L}$  sample volumes and mix. After a 10 minute incubation at room temperature, add 5  $\mu\text{L}$  of developer and read the fluorescence (Figure 1). The assay has an optimal range of 20–500 pmol nitrite (Figure 2), making it up to 50 times more sensitive than colorimetric methods utilizing the Griess reagent. Nitrates may be analyzed after quantitative conversion to nitrites through enzymatic reduction<sup>1</sup>; used in this manner, the assay provides effective quantitation of nitric oxide. The assay is performed at room temperature, and the signal is stable for at least 3 hours. Common contaminants are well tolerated in the assay. The kit provides sufficient material for 2,000 assays.

### Materials

#### Kit Contents

- **Component A:** Measure-iT™ nitrite quantitation reagent, 2.0 mL of a 100X concentrate in 0.62 M HCl
- **Component B:** Measure-iT™ nitrite quantitation developer, 10 mL of 2.8 M NaOH
- **Component C:** Measure-iT™ nitrite quantitation standard, 1 mL of 11 mM sodium nitrite

Sufficient materials are supplied for 2,000 assays, based on a 100  $\mu\text{L}$  assay volume in a 96-well microplate format. The Measure-iT™ nitrite assay can also be adapted for use in cuvettes or 384-well microplates.

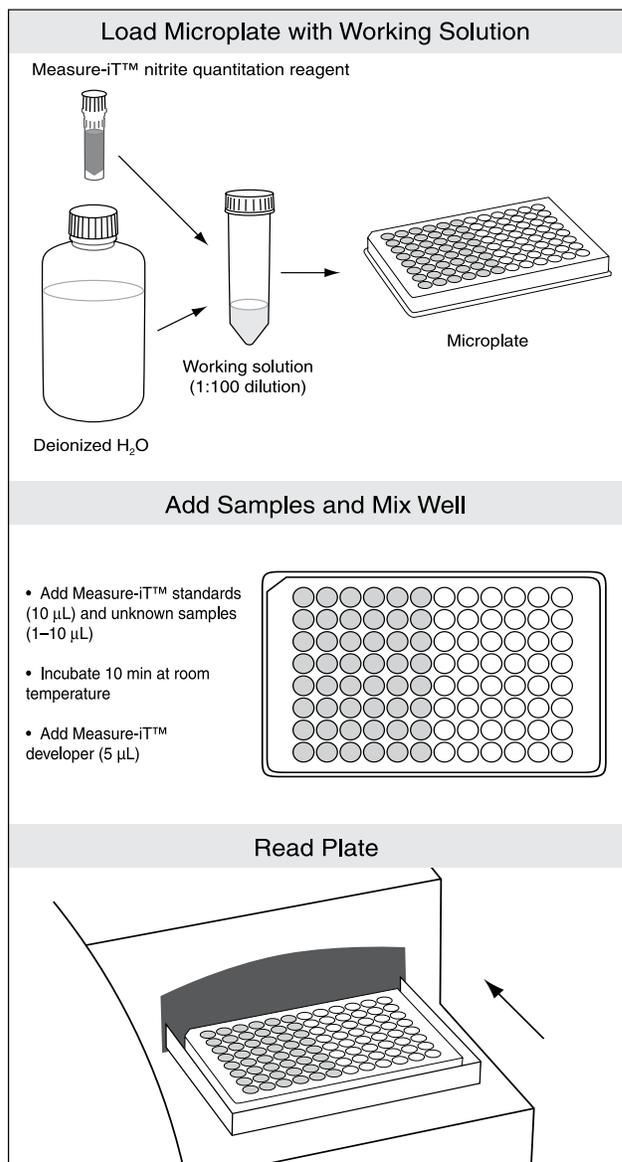
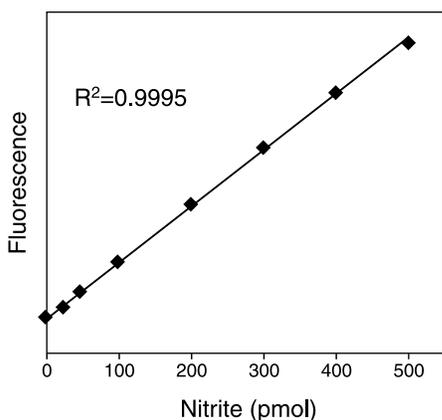


Figure 1. The Measure-iT™ High-Sensitivity Nitrite Assay.



**Figure 2.** Linearity and sensitivity of the Measure-iT™ High-Sensitivity Nitrite Assay. Triplicate 10  $\mu\text{L}$  samples of nitrite were assayed; fluorescence was measured at 365/450 nm and plotted versus picomoles of nitrite. Background fluorescence was not subtracted. The variation (CV) of replicate samples was <2%.

## Storage

Upon receipt, store the kit at  $\leq -20^{\circ}\text{C}$ , protected from light. Under these conditions the components should be stable for at least 6 months. For convenience, Measure-iT™ nitrite quantitation reagent may be stored indefinitely at room temperature, protected from light.

## Handling and Disposal

We must caution that no data are available addressing the toxicity of the Measure-iT™ nitrite quantitation reagent. Treat the reagent with the same safety precautions as all other chemicals with unknown toxicity, and dispose in accordance with local regulations.

## Experimental Protocol

### General Considerations

During all steps, protect the Measure-iT™ nitrite quantitation reagent concentrate and the working solution from light as much as possible. Allow the kit components to equilibrate to room temperature before use. The assay temperature is “room temperature,” defined here as  $20\text{--}25^{\circ}\text{C}$ . Assay temperatures outside this range have not been tested but may be acceptable.

### Nitrite Assay Procedure

**1. Dilute the Measure-iT™ nitrite quantitation standard 100-fold with deionized H<sub>2</sub>O.** For example, dilute 50  $\mu\text{L}$  of the Measure-iT™ nitrite quantitation standard (Component C) with 4.95 mL deionized H<sub>2</sub>O to prepare 5 mL of 110  $\mu\text{M}$  nitrite standard stock solution. This solution is stable for at least 1 week when stored at  $\leq 6^{\circ}\text{C}$ ; storage at  $\leq 4^{\circ}\text{C}$  is recommended.

**2. Prepare Measure-iT™ nitrite quantitation standard dilutions.** Dilute the 110  $\mu\text{M}$  nitrite solution prepared in step 1 with deionized H<sub>2</sub>O according to Table 1. We recommend preparing fresh nitrite standards every 7 days; however, the nitrite standards may remain stable for longer periods when stored at  $\leq 4^{\circ}\text{C}$ .

**3. Prepare a working solution of the Measure-iT™ nitrite quantitation reagent.** For example, for  $\sim 100$  assays, dilute 100  $\mu\text{L}$  of Measure-iT™ nitrite quantitation reagent (Component A) with 10 mL deionized H<sub>2</sub>O in a disposable plastic container and mix well. For best results, briefly agitate the Measure-iT™ nitrite quantitation reagent before use.

**4. Load 100  $\mu\text{L}$  of the working solution into each microplate well.** Diluted Measure-iT™ nitrite quantitation reagent is stable for at least 3 hours at room temperature, protected from light.

**5. Add 10  $\mu\text{L}$  of each nitrite standard prepared in Step 2 to separate wells and mix well.** Duplicates or triplicates of the standards are recommended.

**6. Add 1–10  $\mu\text{L}$  of each unknown nitrite sample to separate wells and mix well.** Duplicates or triplicates of the unknown samples are recommended. Some contaminating substances may interfere with the assay (see below). For highest precision, the volumes of all reactions can be equalized by adding a small volume of the dilution buffer. Equalizing the volumes is especially important in cases where contaminating substances may be present.

**7. Incubate at room temperature and add developer.** Incubate the microplate at room temperature for 10 minutes and add 5  $\mu\text{L}$  of Measure-iT™ nitrite quantitation developer (Component B) to each well.

**8. Measure the fluorescence using a microplate reader (excitation/emission maxima are 365/450 nm).** The fluorescence signal is stable for at least 3 hours at room temperature.

**9. Use a standard curve to determine the unknown nitrite concentration.** For the nitrite standards, plot the nitrite concentration vs. fluorescence and fit a straight line to the data points.

**Table 1.** Preparation of Measure-iT™ Nitrite Quantitation Standards.

Concentration of Nitrite Standard ( $\mu\text{M}$ )	Volume of Nitrite Stock Solution* ( $\mu\text{L}$ )	Volume of Deionized H <sub>2</sub> O ( $\mu\text{L}$ )
0	0	1,000
2.75	25	975
5.5	50	950
11	100	900
22	200	800
33	300	700
44	400	600
55	500	500

\* 110  $\mu\text{M}$  nitrite standard stock solution prepared in step 1.

**Table 2.** Picomole-to-Concentration Conversion.

Picomoles Nitrite	Final Concentration ( $\mu\text{M}$ )	Concentration ( $\mu\text{M}$ ) of Nitrite in Given Sample Volume		
		1 $\mu\text{L}$	5 $\mu\text{L}$	10 $\mu\text{L}$
5	0.5	5	1	0.5
100	1	100	20	10
500	5	500	100	50

\* The Measure-iT™ High-Sensitivity Nitrite assay is designed to detect 20–500 pmol of nitrite in a 100  $\mu\text{L}$  assay volume. Sample volumes may vary from 1–10  $\mu\text{L}$ ; therefore, sample concentration may vary from 0.5–500  $\mu\text{M}$ .

## Protocol Details

### Generating Standard Curves and Extending the Assay Range

In this manual we have plotted standard curves as picomoles of nitrite vs. fluorescence. Alternatively, the x-axis can be expressed in units of molarity or as the concentration of the added sample. Table 2 is provided to facilitate these unit conversions.

The assay is linear from 20–500 pmol nitrite (Figure 2). For best results at the low end of the standard curve, the line should be forced through the background point (or through zero if background has been subtracted). When prepared as described above, the lowest nitrite-containing standard represents 25 pmol of nitrite.

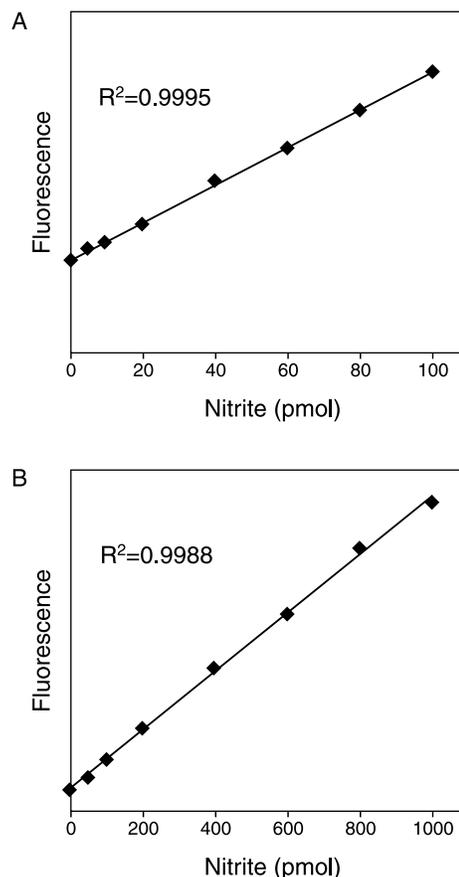
To assess the reliability of the assay in the low range, use smaller volumes of the standards (e.g. 2  $\mu\text{L}$ ) to yield a standard curve ranging from 0–100 pmol (Figure 3). If desired, the utility of the assay can be extended up to 1,000 pmol. For assays in this range, use 20  $\mu\text{L}$  of the prepared standards instead of the 10  $\mu\text{L}$  used above.

### Contaminating Substances

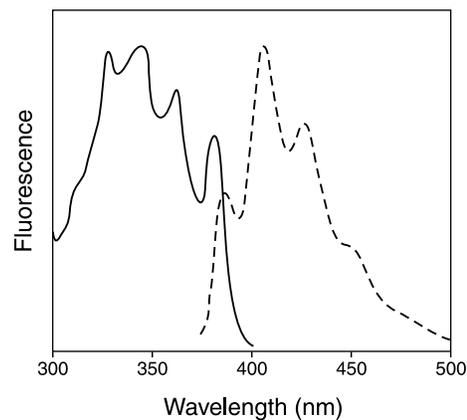
A number of common contaminants have been tested in the Measure-iT™ High-Sensitivity Nitrite assay, and most are well tolerated (Table 3). For untested contaminating substances, and for highest accuracy, the standards should be assayed under the same conditions as the unknown samples. For example, if the experimental samples are in a non-standard buffer and if 10 mL volumes of these samples are used, then add 10 mL of the non-standard buffer (lacking nitrite) when assaying the standards.

### Excitation and Emission Maxima

The excitation and emission spectra for the Measure-iT™ nitrite quantitation reagent bound to nitrite under assay conditions are shown in Figure 4. Excitation bands appear at 328, 346, 362, and 381 nm. The emission spectrum exhibits 3 major peaks of fluorescence intensity at 388, 407, and 427 nm, with a prominent shoulder at 450 nm. Excitation and emission wavelengths of 365 and 450 nm, respectively, are used since they are among the most common commercially available filters. During development of the Measure-iT™ High-Sensitivity Nitrite assay, increased sensitivity was not achieved by using an emission wavelength closer to 410 nm on a monochromator system.



**Figure 3.** Extended Range for the Measure-iT™ High-Sensitivity Nitrite Assay. Triplicate 2  $\mu\text{L}$  (Panel A) or 20  $\mu\text{L}$  (Panel B) samples of nitrite were assayed using the Measure-iT™ High-Sensitivity Nitrite Assay. Fluorescence was measured at 365/450 nm and plotted versus picomoles of nitrite. Background fluorescence was not subtracted. The variation (CV) of replicate nitrite samples for both 2  $\mu\text{L}$  and 20  $\mu\text{L}$  volumes was <6%.



**Figure 4.** Normalized excitation and emission maxima for the Measure-iT™ nitrite quantitation reagent bound to nitrite.

**Table 3.** Effect of Contaminants in the Measure-iT™ High-Sensitivity Nitrite Assay.\*

Contaminant	Final Concentration in the Assay	Concentration in 10 µL Sample	Concentration in 5 µL Sample	Result
Tris, EDTA (pH 7.5)	10 mM/1 mM	100 mM/10 mM	200 mM/20 mM	OK
BSA	10 µg/mL	100 µg/mL	200 µg/mL	NR
BSA	1 µg/mL	10 µg/mL	20 µg/mL	OK
Ascorbic acid	1 µM	10 µM	20 µM	NR
D-(+)-Glucose	10 µM	100 µM	200 µM	OK
DTT	10 µM	100 µM	200 µM	OK
Azide	10 µM	100 µM	200 µM	OK
Cysteine	10 µM	100 µM	200 µM	OK
Glutathione	10 µM	100 µM	200 µM	OK
NaCl	5 µM	50 µM	100 µM	OK
ATP	10 µM	100 µM	200 µM	OK

\* Nitrite standards were assayed in the presence or absence of contaminants at the indicated final concentrations. Equivalent concentrations (approximate) in 10 µL or 5 µL sample volumes are also listed. Results are given either as OK = usually less than 10% perturbation, or as NR = not recommended.

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## Reference

- Anal Biochem 126, 131 (1982).

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## Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
M36051	Measure-iT™ High-Sensitivity Nitrite Assay Kit *2000 assays* .....	1 kit

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