SKU: NaRA-10

NADH:Nitrate Reductase (NaR) Activity Assay Kit

Reagents and Instructions to extract 10 plant tissue samples
- Reagents and Instructions for assay of NaR in plant extracts
  - Purified NaR activity standard included with the kit

OVERVIEW

- ✓ **Store kit refrigerated** or below 60°F (15°C).
- ✓ *See box for expiration date.*
- ✓ **Store all reagents as directed once kit has been opened!**

This kit will provide reliable estimates of NaR activity when used as directed.

♦ EQUIPMENT AND REAGENTS

You will need to supply:

- ❑ distilled or deionized water (d-I water)
- ❑ 3 N HCl
- ❑ Phosphate buffer for plant extractions (recipe provided with kit)
- ❑ pipets, vortex mixer
- ❑ water bath set at 30°C
- ❑ colorimeter with 540 (±20) nm filter
- ❑ tubes for 4 mL assay
- ❑ centrifuge for assay tubes

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This Kit enables you to prepare extracts of plant materials and to assay these extracts for NADH: Nitrate Reductase (NaR) activity. The enzyme Nitrate Reductase (NaR) catalyzes the reduction of Nitrate to Nitrite using the natural electron donor NADH. The nitrite reacts with color reagents (dyes) under acidic conditions to produce a visible magenta color. The color produced is directly proportional to the nitrate concentration. The quantity of nitrate reduced in the reaction time tells you how much active nitrate reductase is present in your sample.

The Enzyme Commission definition of NaR activity is expressed as micromoles of nitrite produced per minute per ml of enzyme (µmol nitrite formed/ min/ ml of extract), in a buffer at pH 7.5 at 30°C. We provide a Nitrate Reductase Activity Standard (YNaR) at 1.0 Unit per milliliter (1.0 U reduces one µmol nitrate per minute). You will use this YNaR standard to determine the NaR activity in your samples.

Supplied in NECi Test Kit

- ❑ **NaR Assay Buffer** in liquid form – four 25 ml tubes
- ❑ **Nitrate Solution (500 mM K-Nitrate)** in liquid form – two 1.5 ml tubes
- ❑ **YNaR, Nitrate Reductase** in freeze-dried form, in foil pouch
- ❑ **Enzyme Diluent bulb** for reconstitution and storage of YNaR
- ❑ **Color Reagent No. 1** in solid form - one 60 ml amber bottle
- ❑ **Color Reagent No. 2** in solid form – one 60 ml amber bottle
- ❑ **NADH** in freeze-dried form - four tubes in amber bag
- ❑ **Zinc Acetate** in solid form - one 5 ml vial
- ❑ **Reagents for extraction of NaR from your plant samples:**
  - ❑ **PVPP –** polyvinylpolypyrrolidone
  - ❑ **Cysteine –** L-cysteine

Supplied by User

- ❑ **Labware for making up reagent solutions**
- ❑ **Grinder, blender, or mortar and pestle** for preparing plant extracts
- ❑ **Variable pipettors** (100 to 1000 µl and up to 2 ml).
- ❑ **Test tube vortex-type mixer** or other means to mix contents of tubes.
- ❑ **Water bath** for running reaction at 30°C
- ❑ **Colorimeter or Spectrophotometer** capable of reading at 540 nm ± 20 nm, with a glass or plastic cuvette (approx. volume 2 ml).
- ❑ **Test tubes** (Clean and Nitrate-free).
- ❑ **Timer** (0 to 20 minutes) – a clock or stop watch is adequate.
- ❑ **Deionized or distilled water** (d-I water; must be "Nitrate-free" to avoid high background).
- ❑ **3 N or concentrated HCI (hydrochloric acid)** used to make Color Reagent #1.
- ❑ **Ice and Ice Bucket**
- ❑ **Centrifuge for assay tubes.**

NEED HELP? Contact NECi
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♦ REAGENT PREPARATION

Store Assay Buffer and Color Reagents at 4°C between uses.

Store YNaR standard and NADH solutions at -20°C.

NOTES ON THE REAGENTS

CAUTION: Color Reagent #1 is made with HCl! Use standard precautions for handling acids. Pipet with care.

Zinc Acetate solution is corrosive. Don't leave the wet tip on your pipettor.

1. **NaR Assay Buffer:** Add nitrate before use! Warm to room temperature and add 1 ml of Nitrate Solution to 50 mL of buffer, and mix. Prepare 50 ml of NaR Assay Buffer + Nitrate at a time. (Use 2 x25mL buffer.) Repeat as needed. Store at 4°C.

2. **Color Reagent #1:** Prepare **3 N HCI** by adding **15 ml concentrated HCI** to **45 ml d-I water.** Mix. Add **3 N HCI** to **Color Reagent No. 1** bottle. Mix by inverting several times until all the solid dissolves. Store at 4°C.

3. **Color Reagent #2:** Add **60 ml d-I water** to **Color Reagent No. 2** bottle. Mix by inverting several times until all the solid dissolves. Store at 4°C.

4. **NADH solution:** Remove 1 tube of **NADH** from amber bag, tap tube to settle contents. Add **1.5 ml d-I water** and replace cap. Mix gently with vortex-type mixer. Keep on ice during use. Freeze between uses.

5. **YNaR nitrate reductase standard solution:** Twist end off **Enzyme Diluent bulb** and squeeze contents into **YNaR** vial. Let stand 10 minutes, mix gently at 5 minute intervals. Keep on ice during use and store at -20°C.

6. **Zinc Acetate:** Add 5.0 ml d-I water to **ZnAc vial** (plastic bottle with white cap)

Final concentrations of Reagents:

- **Assay Buffer** - 25 mM KH_2PO_4, 0.025 mM EDTA; pH 7.5.
- **Assay Buffer will contain 10 mM Nitrate** after KNO_3 is added**.**
- **Color Reagent No. 1** - 1% Sulfanilamide in 3 N HCl.
- **Color Reagent No**. **2** - 0.02% N-Naphthylethylenediamine in d-I water.
- **NADH** – approx. 2 mM NADH – once the dry form is in solution.
- **YNaR** – Nitrate reductase, 1.0 U/ml
- **ZnAc** – 1 M Zinc acetate

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♦ PREPARATION of PLANT EXTRACTION BUFFER

We do not supply the plant extraction buffer.

Additives to protect nitrate reductase activity ARE supplied in the kit. Add these to your buffer as directed here.

Store buffer at 4°C and use cold for extraction.

Nitrate Reductase Extraction Buffer:

We recommend 0.1 M Potassium Phosphate containing 1 mM EDTA, pH 7.5. Use Reagent Grade or better. ***You can substitute a sodium phosphate or MOPS buffer. DO NOT use Tris buffer.***

RECIPE FOR ONE LITER OF EXTRACTION BUFFER:
13.6 g KH_2PO_4 (potassium phosphate, monobasic)
370 mg Na_2EDTA (EDTA, sodium salt)
5 g KOH (potassium hydroxide)

Make up to 1 liter using d-I water, mix thoroughly and adjust pH. The pH will be about 7.5, but be sure to check and adjust as necessary. You don't need to be exact here. Volume should be 1 liter ± 10%, pH range between 7.0 – 7.8 is OK for nitrate reductase.

We recommend a ratio of four milliliters of extraction buffer per gram of plant tissue (wet weight). We recommend using 10 grams of sample per extraction, if enough material is available, so that you have a representative sample. You will add the PVPP and Cysteine when you are ready to grind the tissues in the extraction buffer.

Nitrate reductase is a very sensitive and unstable enzyme. The PVPP will protect the enzyme from phenolic compounds. The Cysteine protects sulfhydryl groups that are critical for enzyme structure. We do NOT provide any protease inhibitors with this kit. If your sample type is known to have strong protease activity, consult the literature for the appropriate inhibitor and add to the extraction buffer immediately before use.

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PLANT EXTRACTION PROCEDURE

For best results, keep everything cold: use an ice bucket, start with solutions at 4°C, work in a cold room. Keep solutions and vessels in a refrigerator until use.

We recommend 10 grams of plant material to 40 ml of extraction buffer. If sample size is limited, reduce all ingredients proportionately to suit your sample size.

For quantitative results, measure the volume of each plant extract. Use this value in Calculations on Page 4.

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NaR Activity Assay using YNaR standard

This section is *optional.* We include it to allow you to practice the NaR assay.

Have Assay Buffer at room temp before you begin the assay procedure. Saves time!

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NaR ACTIVITY ASSAY

Here's where you assay the NaR activity in your plant extracts.

Part I: nitrate reduction

Have water bath ready at 30°C. Have Assay Buffer at room temp.

- There are many methods for preparation of plant extracts. A standard kitchen or lab blender, or mortar and pestle may be used. It is important to have all containers and reagents cold when you begin the extraction. This will help preserve enzyme activity.
- Start with freshly cut plant materials whenever possible. If samples must be shipped and/or stored, use dry ice or store at -70°C. Liquid nitrogen is also fine.
- Suggested protocol:
  10 g of your plant material (freshly cut or frozen)
  40 ml chilled Extraction Buffer (prepared above)
  0.4 g PVPP (provided with kit)
  0.03 g L-Cysteine (provided)

1. **Combine** ingredients into a chilled blender vessel and grind until mixture consists of a slurry of uniform small particle size – approx one minute at high speed. Be sure the PVPP and Cysteine are mixed into the slurry – PVPP will not go into solution.
2. **Filter** the slurry through 2-4 layers of cheesecloth, Miracloth or equivalent.
3. **Optional: Centrifuge** at 4°C 10,000 rpm/10 min
4. **Repeat** for each sample. **Keep samples on ice** until ready to assay. Do not freeze!

Alternate: Use mortar and pestle. The extraction will be more uniform if you start with frozen materials. Dry ice or liquid nitrogen may be used. Follow steps as listed above.

Note: You can use smaller quantities of plant material if sample is limited. Retain the ratio of 4 volumes of Extraction Buffer to gram of plant material, and reduce the PVPP and Cysteine proportionately. A little too much of either does no harm.

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Nitrate Reductase Activity assay using Purified YNaR Standard

We provide this protocol as a reality check. Test that all assay reagents are ready to use and get some familiarity with the NaR assay before you waste any precious samples!

1. Label 4 tubes: #1 is your Reagent Blank. Set tubes in water bath at 30°C.
2. Add **1.8 ml Assay Buffer** to each tube. Allow enough time for Assay Buffer to reach 30°C.
3. Add **100 µl YNaR** solution to each tube. Mix. Do not add NADH to Tube #1!
4. This is where the timing begins, so have your stopwatch or clock in eyesight. **Add 100 µl NADH to tubes #2 – 4. Note exact time you add the NADH to each tube! Mix gently.** Leave 10 – 15 seconds between tubes to give yourself time for Step 5. The YNaR will begin to reduce nitrate the instant NADH is added!
5. **At exactly one minute (60 seconds) STOP the reaction by adding 1ml of Color Reagent #1 to EACH tube. Mix. (**This is why it is important to stagger the addition of NADH in Step 4.)
6. **Add 1ml Color Reagent #2 to all tubes. Mix.**
7. **Read absorbance at 540nm.** Absorbance should be **low or Zero for tube #1** (the Blank), and **approx 1.0 for tubes #2-4** (the YNaR standard). If absorbance is higher than 0.030 for the Blank, there is nitrate or nitrite in your water, glassware, or something else in your lab.

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Assay Nitrate Reductase Activity in your Samples

Part I: nitrate reduction step

These steps allow the enzyme to react and remove protein interferences.

1. For each sample to be analyzed:
   a. Label 4 microfuge tubes. #1 = Blank; #2 = 5 minute time point, #3 – 10 min, #4 = 15 min. *This allows you to get a useable result when NaR activity is low.* **Set tubes** in water bath at 30°C.
   b. Add **1.8 ml Assay Buffer** to each tube.
   c. Add **100 µl of your plant extract** to each tube. **Mix.**
   d. Set **#1**, the Blank, aside
2. This is where timing begins, so have your stopwatch or clock in view. **Add 100 µl NADH to tubes #2-4. Mix. Allow each tube to react** for correct number of minutes.
3. **Stop reaction** by adding **100 µl ZnAc** to tube **#2 at 5** min, **#3 at 10** min, **#4 at 15** min. Add ZnAc to tube #1 at any time. Mix thoroughly after adding the ZnAc.
4. **Centrifuge** tubes to remove precipitate. Two – 5 min at 5000 rpm, longer at slower rpm.

NaR ACTIVITY ASSAY PART II: COLOR DEVELOPMENT AND CALCULATIONS

WASTE DISPOSAL
Follow all local guidelines and regulations. If there are no local guidelines, wash the waste down the sink with large amounts of running water.

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Assay Nitrate Reductase Activity in your samples, Part II:

Color Development

5. **Pour supernatant** from Step 4 tubes into clean, labeled tubes.
6. **Add 1ml Color Reagent #1** to each tube**. Mix.**
7. **Add 1ml Color Reagent #2** to each tube**. Mix.** Allow 10 minutes for color to develop fully. The **Blank** tube should be nearly colorless.
8. **Read absorbance** at 540 nm (± 20nm). Use d-I water to zero the colorimeter or spectrophotometer. Rinse cuvette with d-I water between samples.

Calculations:

1. **Deduct** the absorbance you got for your **Blank** from the absorbance of the samples. This corrects for any nitrite that may be present in your plant extracts.
2. **Divide** each time point to get absorbance per minute.
   a. **Example: (**A_{540} tube #2 – A_{540} tube #1)/5 min

3. **Average** the time points from all three tubes. Reject any data points with absorbance higher than 1.5AU. This gives you average change in absorbance per minute.

4. **Now you can determine NaR activity in Units/ml:**

   Average change in A_{540}/min = Units/ml

   Example: If average A_{540}/min is 0.014, then Units/ml = 0.014 U/ml

5. **NaR activity is generally expressed as µmol nitrite produced per hour per gram fresh weight of plant material** in plant extracts. Here's how to calculate µmol/hr/gfw: For the example above, take the original total volume of the plant extract (assume ~45ml if you haven't measured it):

   45 ml(0.014 U/ml) = 0.63U total in the plant extract

   $$\frac{\text{(Total units)(60 min)}}{\text{grams plant material}} = \frac{0.63(60)}{10\text{ g}} = 3.78\ \mu\text{mol/hr/gfw}$$

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NOTES: If your NaR source has **high NaR activity**, your A_{540} for the longer time points will be > 1.5. In that case, use shorter time points (e.g 1,2,3 minutes). If your A_{540} values are too low, your plant materials have **low NaR** activity. In such cases, increase your time points (10,20,30 min).

To test whether your extracts inhibit NaR: If you consistently see little or no NaR activity, there may be proteases or inhibitors of NaR in your plant extracts. An easy test of this is to add 100 µl YNaR standard to one of your extracts (NaR "spike"). Do a **one minute** assay as directed on the bottom section of Page 3. If you do not see A_{540} of at least 1.0, something in your extract inhibits or destroys NaR. Consult the literature for NaR assays for your specific plant material. You may need to add protease inhibitors, FADH, Dithiothreitol or other additives during your extraction procedure.

This kit will NOT detect NADPH NaR Activity unless you substitute your own NADPH for the supplied NADH.

Thanks for using our products. Call Tech Support: 1.906.296.1130, or visit the NECi website: www.nitrate.com if you need more information. We're always interested in hearing about your experience with our kits.

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