Chapter 13

Analysis of the Expression and Activity of Nitric Oxide Synthase from Marine Photosynthetic Microorganisms

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Abstract

Nitric oxide (NO) functions as a signaling molecule in many biological processes in species belonging to all kingdoms of life. In animal cells, NO is synthesized primarily by NO synthase (NOS), an enzyme that catalyze the NADPH-dependent oxidation of l-arginine to NO and l-citrulline. Three NOS isoforms have been identified, the constitutive neuronal NOS (nNOS) and endothelial NOS (eNOS) and one inducible (iNOS). Plant NO synthesis is complex and is a matter of ongoing investigation and debate. Despite evidence of an Arg-dependent pathway for NO synthesis in plants, no plant NOS homologs to animal forms have been identified to date. In plants, there is also evidence for a nitrate-dependent mechanism of NO synthesis, catalyzed by cytosolic nitrate reductase. The existence of a NOS enzyme in the plant kingdom, from the tiny single-celled green alga *Ostreococcus tauri* was reported in 2010. *O. tauri* shares a common ancestor with higher plants and is considered to be part of an early diverging class within the green plant lineage.

In this chapter we describe detailed protocols to study the expression and characterization of the enzymatic activity of NOS from *O. tauri*. The most used methods for the characterization of a canonical NOS are the analysis of spectral properties of the oxyferrous complex in the heme domain, the oxyhemoglobin (oxyHb) and citrulline assays and the NADPH oxidation for in vitro analysis of its activity or the use of fluorescent probes and Griess assay for in vivo NO determination. We further discuss the advantages and drawbacks of each method. Finally, we remark factors associated to the measurement of NOS activity in photosynthetic organisms that can generate misunderstandings in the interpretation of results.

Key words Nitric oxide, Nitric oxide synthase, Oxyhemoglobin assay, Citrulline detection, Griess assay, DAF-FM diacetate

1 Introduction

Nitric oxide synthases (NOSs, EC 1.14.13.39) are present in insects, mollusks, parasites, fungi, slime molds, bacteria and recent genome sequencing revealed that NOS proteins exist in photosynthetic organisms [1–5]. Their amino acid sequences and activities are similar to the mammalian NOSs, suggesting that the mammalian gene came from lower species through evolution [6]. The prokaryotic forms have been considered to be the precursors of animals

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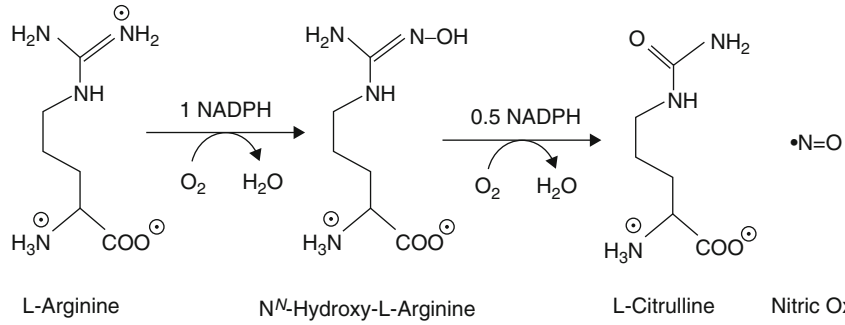
NOS. The last were originated during evolution by the fusion of a bacterial oxygenase domain to a dedicated reductase domain. The bacterial enzymes generally have only the oxygenase domain highly homologous to the mammalian counterpart. However, the NOS from the bacterium *Sorangium cellulosum* contains a reductase domaintogether the oxygenase, with a different arrangement of the domains between bacterial and animal NOS enzymes, suggest independent events in prokaryotic and eukaryotic lineages [4, 7].

The mammalian NOSs catalyze the oxidation of l-Arginine to l-citrulline and NO, with *N*-hydroxy-l-Arg formed as an enzyme-bound intermediate as shown in [A].



All mammalian NOSs are bi-domain proteins comprising an N-terminal oxygenase domain (NOSoxy) that binds protoporphyrin IX (heme), 6*R*-tetrahydrobiopterin (BH_4), and Arg, and a C-terminal flavoprotein domain (NOSred), linked together by a calmodulin (CaM) binding sequence. NOS flavoprotein domains are similar to NADPH-cytochrome P450 reductase and related electron transfer flavoproteins, and function to provide NADPH-derived electrons to the ferric heme for O_2 activation during NO synthesis [8].

The identification of the NOS from the green algae *O. tauri* represents the first NOS characterized in the plant kingdom [5]. By sequence homology analysis, more NOS sequences can be found in other photosynthetic organisms. *O. lucimarinus*, another species of the Ostreococcus genus, also contains a NOS gene, the expression of which was validated by EST analysis [5]. In *Bathycoccus prasinos* genome there is a sequence with 75 % (accession number XP_007510938) and in *Thalassiosira oceanica* genome (accession number EJK55330) a sequence with 56 % similarity with the *Ostreococcus tauri* NOS (OtNOS), indicating that novel NOS proteins will be discovered in the plant kingdom in the future. The big challenge remaining undeciphered is to identify and characterize the NOS activity in higher plants.

As sated before, OtNOS is the unique NOS enzyme characterized in photosynthetic organisms [5]. OtNOS has a 42 % similarity to human NOS reaching to 45–49 % similarity to invertebrate

NOS. OtNOS contains the NOSoxy and NOSred domains joined by a CaM binding domain. Despite the high similarity, some differences could be noted in the structure of the OtNOS with respect to animal NOS. CaM plays a critical role in activating NOS, since it triggers the electron transfer from flavin to heme. OtNOS activity behaves like an intermediate between eNOS/nNOS and iNOS (endothelial, neuronal and inducible NOS respectively) isoforms since in the absence of Ca^{2+}-CaM, OtNOS retains almost 70 % of activity. Furthermore, OtNOS lacks of the autoregulatory control element (ACE) [5], indicating that it is close to the mammalian iNOS isoform. The ACE impedes CaM binding and enzymatic activation in constitutive NOSs (eNOS and nNOS). The increase in Ca^{2+} concentration triggers the binding of Ca^{2+}-CaM in constitutive NOSs by displacing the ACE [9]. BH_4 cofactor is essential for NO production in animals since the absence of BH_4 uncouples the reaction leading to NADPH oxidation and superoxide formation [10]. Ostreococcus genome has been completely sequenced [11] and it lacks the genes encoding for the enzymes that synthesize BH_4, suggesting that OtNOS may bind another cofactor for catalytic activity. Thus, it is potentially a useful model system to study gene evolution and cellular processes in photosynthetic eukaryotes.

A good approach for the characterization of a canonical NOS enzyme is the recombinant expression of the NOS protein in *Escherichia coli*. This strategy allows to express and purify the protein for analyzing its spectral characteristics and in vitro activity. Furthermore, in *E. coli* expressing the recombinant NOS protein it can be measured NO production and nitrite levels, an indirect method used to quantify NO concentration in vivo. Increased NO and nitrite levels were detected in *E. coli* expressing NOS recombinant enzyme from mammalian, bacterial, and photosynthetic organisms [5, 6]. However, the detection of the NOS activity in vivo in the original organism still remains essential. Foresi et al. [5] reported the functional characterization of the NOS enzyme from *O. tauri* by heterologous expression in *E. coli*. Bacterium carrying the NOS gene displayed enhanced NO production and cell viability. In *O. tauri*, OtNOS protein was detected throughout its life cycle participating in light-regulated response of the algae. In this protocol, the methodology used to study expression and the activity of NOS in the green algae will be described and discussed in detail.

2 Biological Materials

Cloning, expression and purification of heterologous expression of recombinant NOS in *E. coli* is a useful approach to study the NOS activity in vitro and in vivo.

2.1 Cloning of Recombinant NOS

1. Synthesizing, sequencing and cloning the full length NOS sequence or the oxy domain (CaM domain, inclusive) of NOS into pET expression system vector (*see* **Note 1**).
2. Transform competent BL21 (DE3) protease-deficient *E. coli* cells with pET-NOS (*see* **Note 2**).

2.2 Recombinant NOS Expression

2.2.1 NOSoxi Expression

1. Inoculate Erlenmeyer flasks containing 0.5 L of modified Terrific Broth (20 g of yeast extract, 10 g of bacto tryptone, 2.65 g of KH_2PO_4, 4.33 g of Na_2HPO_4, and 4 mL of glycerol) and ampicillin (125 μg/mL) with 500 μL of culture pET15b-NOS and grown overnight and then shaking at 150 rpm at 37 °C.
2. Induce the recombinant protein expression at OD_{600} between 1 and 1.2 by the addition of 1 mM IPTG (*see* **Notes 3** and **4**). Add the heme precursor 8-aminolevulinic acid at final concentrations of 500 μM.
3. Harvest the cells after 72 h at 20 °C (*see* **Note 4**) of induction and resuspend in 250 mL of Lysis buffer (100 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mg/mL lysozyme, 10 % [v/v] glycerol, 1 mM PMSF, 1 mg/L leupeptin, and 0.5 mg/L pepstatin, 5 mg/L aprotinin, 10 mM l-Arg, 10 μM BH_4 in ascorbic acid, 50 U/mL of DNAse). Lysis is achieved by cell disruption using a Cell disruptor Brand or a French press at 0.6/0.8 kbar.

2.2.2 Full NOS Expression

1. Inoculate Fernbach flasks containing 1 L of modified Terrific Broth and kanamycin (50 mg/mL) with 1 mL of culture pET-NOS and grown over night shaking at 190 rpm at 37 °C.
2. Induce the recombinant protein expression at OD_{600} 0.4 by the addition of 0.5 mM IPTG (*see* **Notes 3** and **4**). Add the heme and flavin precursors 8-aminolevulinic acid and riboflavin at final concentrations of 450 and 3 μM, respectively.
3. Harvest the cells after 24 h (*see* **Note 4**) of induction and resuspend in 30 mL of buffer (100 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 10 % [v/v] glycerol, 1 mM PMSF, 5 mg/mL leupeptin, and 5 mg/mL pepstatin) per liter of initial culture and lysed by pulsed sonication (six cycles of 20 s).
4. Analyze the best conditions for NOS expression testing different concentrations of IPTG and time of induction. Recombinant NOS is then studied by SDS-PAGE and Western blot analysis using a specific anti-NOS antibody or anti-H is when this tag is added (*see* **Note 5**).

2.3 NOS Purification

1. For OtNOS oxi domain, remove cell debris by centrifugation (20,000 × *g*). The supernatant is applied on a NTA-Ni^{2+} column (20 mL), previously equilibrated in MCAC buffer (100 mM Tris–HCl, pH 7.4, 10 % glycerol, 150 mM NaCl, 1 mM PMSF, 10 mM l-Arg, 10 μM BH_4 in ascorbic acid). Column is extensively washed (8 column volumes) with a MCAC + 40

mM imidazole buffer. Elution is achieved by the addition of a MCAC + 300 mM imidazole buffer.

2. For full NOS purification remove cell debris by centrifugation, and apply the supernatant to an ADP-agarose 4B column (1 mL) equilibrated in buffer B (50 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM DTT, 10 % glycerol, and 100 mM NaCl) (*see* **Note 6**). Wash the column with 10 column volumes of buffer B and finally with buffer B and 500 mM NaCl. Elute the protein with buffer B, 500 mM NaCl, and 25 mM 2′-AMP (*see* **Note 7**).

3 Methods

Carry out all the procedures at 4 °C temperature, NOS activity is quickly lost at room temperature.

3.1 Spectral Properties of the Oxygenase Domain of NOS

1. Recondition the oxygenase domain in a KPi, pH 7.4 10 % [v/v] glycerol and 150 mM NaCl Buffer by three cycles of concentration/dilution.
2. Analyze the UV–visible absorption spectra (Fig. 1 main) are then recorded for the native enzyme (around 20 μM) and upon successive additions of l-Arginine (10 mM final) and BH_4 (40 μM final). Fig. 1 Inset displays the characteristic spectrum of the $Fe^{II}CO$ complex obtained upon addition of sodium dithionite and CO flushing.

3.2 Determination of NOS Activity In Vitro

3.2.1 Spectro-photometric Method

1. Reduce the hemoglobin to oxyhemoglobin with sodium dithionite (*see* **Note 8**) Determine the oxyhemoglobin concentration in the solution using a molar extinction coefficient of 131 mM^{-1} cm^{-1} at 415 nm.
2. Prepare a 500 μL-mix reaction containing 20 mM oxyhemoglobin, 7.5 mM HEPES-NaOH, pH 7.5, 5 mM DTT, 100 μM l-arginine, 1 μM NADPH, 10 mM $CaCl_2$, 10 μM CaM, 100 μM BH_4, and 100 U/mL catalase.
3. Initiate the reaction by the addition of 0.5 μM purified NOS protein. Monitor the NO-dependent conversion of oxyhemoglobin to methemoglobin on a spectrophotometer by scanning between 380 and 450 nm. Use an extinction coefficient of 100 mM^{-1} cm^{-1} to quantify NO production as the difference between the peak at 401 nm and the valley at 420 nm.

3.2.2 NADPH Oxidation

1. Prepare 500 μL volume containing 0.5 μM NOS, 50 mM Tris–HCl, pH 7.6, 5 mM DTT, 100 μM l-Arg, 10 mM $CaCl_2$, 10 μM CaM, 100 μM BH_4, and 100 U/mL catalase and start the reaction with 1 μM NADPH (*see* **Note 9**).
2. Monitor the rate of decrease in absorbance at 340 nm for 10 min at 25 °C using a spectrophotometer. An extinction

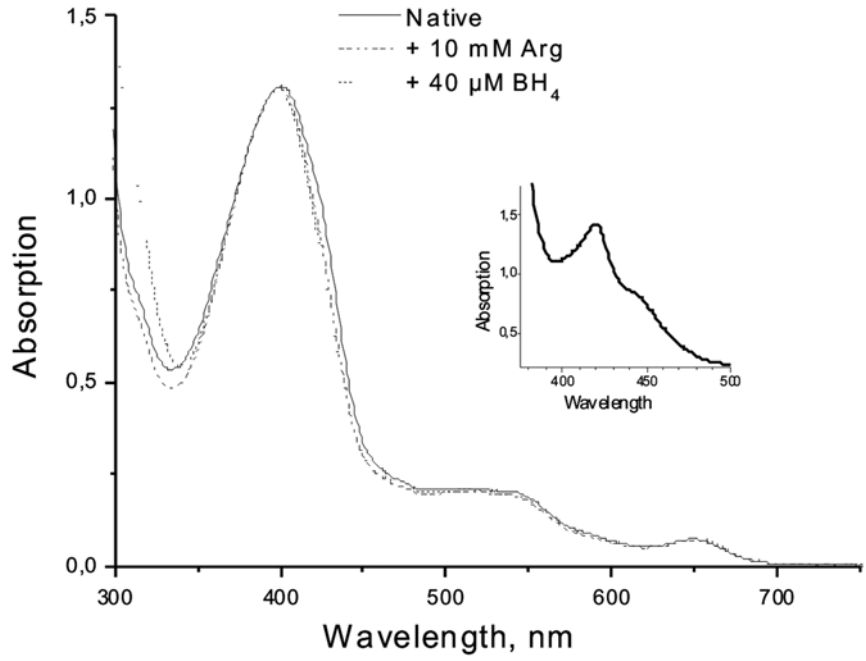


Fig. 1 UV–visible absorption spectra of the oxygenase domain of OtNOS. *Main panel*: Native OtNOS (*solid line*) shows spectroscopic fingerprints charecteristic of mammalian NOSs: a Soret maximum around 402 nm (indicating a mixture of high spin and low spin species), an α/β region with two bands around 515 and 545 nm and a charge transfer band at 650 nm. Addition of the substrate L-arginine (*dashed line*) and the cofactor BH_4 (*dotted line*) shifts the mixture to a fully high spin species (I_{max} at 398 nm). *Inset*: P450 spectra of OtNOS. The $Fe^{II}CO$ complex of OtNOS shows a mixture between P450 and P420 conformations, indicating a certain liability of the proximal ligation

coefficient of 6.22 mM^{-1} cm^{-1} at 340 nm is used to calculate NADPH oxidation.

3.2.3 Citrulline Detection

1. Perform the enzymatic reaction at 25 °C in 50 mM Tris–HCl, pH 7.4, containing 50 μM l-Arg, 1 μCi [3H] l-Arginine monohydrochloride (40–70 Ci/mmol; Perkin-Elmer), 100 μM NADPH, 10 μM FAD, 2 mM $CaCl_2$, 1 μg CaM, and 100 μM BH_4 in a volume of 40 μL (*see* **Note 10**).
2. Initiate enzymatic reactions by adding 0.5 μM NOS and terminated after 5–30 min by the addition of 400 μL of ice-cold 20 mM sodium acetate, pH 5.5, containing 1 μM l-citrulline, 2 mM EDTA, and 0.2 mM EGTA (stop buffer).
3. Apply the sample to columns containing 1 mL of Dowex AG50W-X8, Na^+ form (Bio-Rad; 100–200 mesh), pre-equilibrated in stop buffer.
4. Elute l-citrulline with 2 mL of distilled water. Aliquots of 0.5 mL of eluate are dissolved in 10 mL of scintillation liquid consisting of 0.35 % (w/v) PPO (2,5-diphenyloxazole), 0.03 % (w/v) POPPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene),

1 % (w/v) naphthalene, 2.3 % (v/v) ethanol, 3.85 % (v/v) dioxane, and 3.85 % (v/v) toluene. Radioactivity is measured in a liquid scintillation counter.

For a correct citrulline assay interpretation a thin layer chromatography is necessary (*see* **Note 10**). The citrulline production by OtNOS was evidenced by TLC (Fig. 2a, b).

1. Prepare the reaction: 50 mM Tris–HCl (pH 7.4) containing 50 μM l-Arg, 1 μCi [^{3}H]l-arginine monohydrochloride, 100 μM NADPH, 10 μM FAD, 2 mM $CaCl_2$, 1 μg CaM, and 10 μM BH_4 in a final volume of 40 μL.
2. Start the reaction by the addition of 0.5 μM OtNOS or H_2O (Control).
3. After 30 min at 25 °C, stop the enzymatic reaction with 40 μL of 20 mM sodium acetate pH 5.5, containing 2 mM EDTA and 0.2 mM EGTA, and load onto a Dowex AG 50 W-X8 resin column.
4. Elute the citrulline by centrifugation and separate it by TLC.
5. Run standard solutions contained 0.2 μmol of l-Arg, 0.2 μmol l-citrulline and a mix containing 0.1 μmol of l-Arg plus 0.1 μmol of l-citrulline.
6. Separate the amino acids on Silica-60 TLC plates (Merck) employing chloroform, methanol and ammonium hydroxide (2:3:2, v/v/v) as the mobile phase.
7. Stain the amino acids with ninhydrin solution (0.1 %, w/v, ninhydrin in ethanol–acetic acid, 5:1, v/v).
8. Remove the amino acids spots from TLC plates and determine the radioactivity using a liquid scintillation counter.

3.3 In Vivo Assay Method to Detect NOS Activity

The in vivo NOS activity can be analyzed in the bacteria expressing the recombinant protein.

3.3.1 Griess Assay

1. Determination of NO formation using the Griess reagent in *E. coli* (*see* **Note 11**). For a nitrite measurement inoculate Fernbach flasks with 500 μL of culture containing 50 mL of LB medium and shake at 190 rpm at 37 °C. Induce recombinant protein expression by the addition of IPTG (*see* **Note 4**).
2. Add the heme and flavin precursors 8-aminolevulinic acid and riboflavin, to 450 and 1 μM, respectively, and the substrate l-Arg to 1 mM, all of them final concentrations.
3. The cells are harvested 5 h after the induction and the pellet is solubilized in 100 mM phosphate buffer pH 7.5 and lysated by pulsed sonication (six cycles of 20 s each).
4. Remove cell debris by centrifugation, and use the supernatant for Griess assay. Fifty microliters of sample is placed in a 96-well micro assay plate.

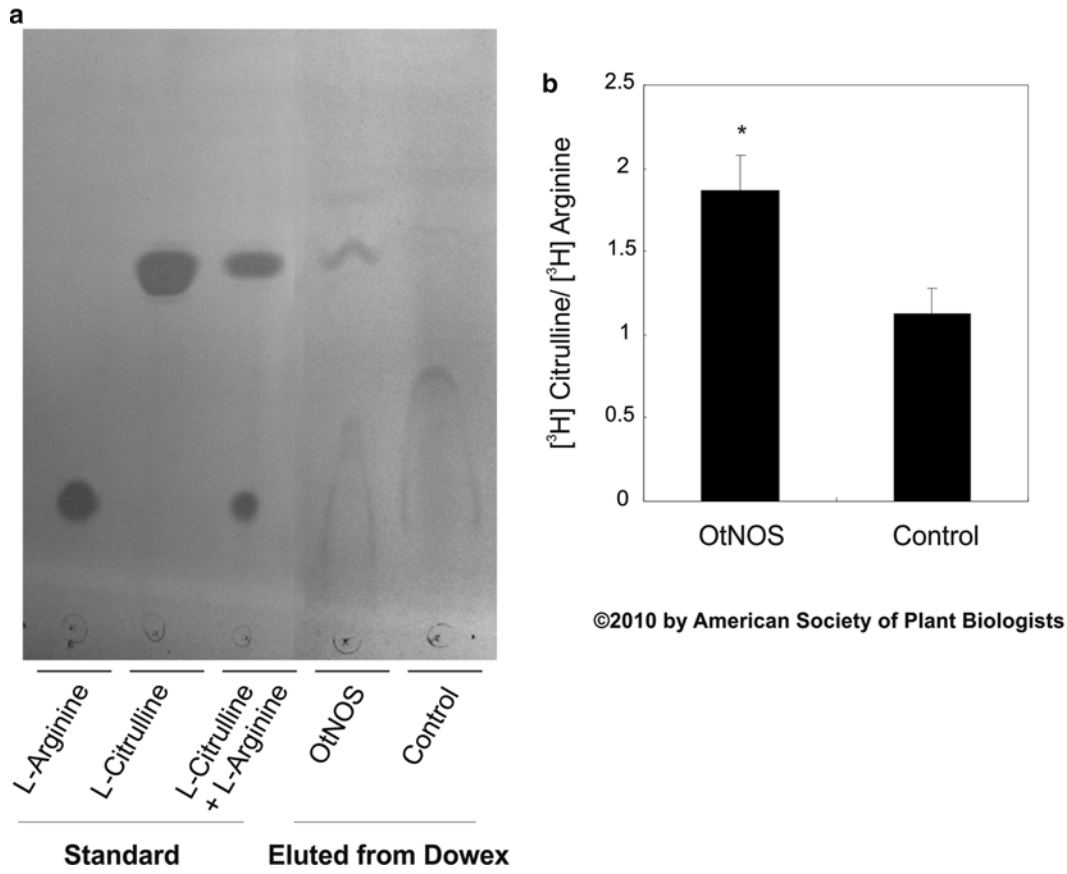


Fig. 2 Detection of L-citrulline as a product of OtNOS enzymatic activity. (**a**) Thin layer chromatography (TLC) of the products of reactions. Retension factors (Rf) were determined (L-arginine, 0.21; L-citrulline, 0.59). (**b**) Amino acids were removed from TLC plates and radioactivity was determined using a liquid scintillation counter. Values are expressed as the ratio of [^{3}H]L-citrulline to [^{3}H]L-arginine. *Error bars* denote SE ($n=4$) and *asterisk* indicates a statistically significant difference (*t* test, $p<0.05$). Foresi et al., 2010. www.plantcell.org. Copyright American Society of Plant Biologists

5. Add 50 µL of sulfanamide in 5 % [v/v] phosphoric acid; incubate for 10 min at 20 °C.
6. Add 50 µL of 1 % (w/v) *N*-(1-naphthyl) ethylenediamine HCl (NED) and incubate for 10 additional min in the dark.
7. Read the absorbance at 550 nm in a microplate reader. A nitrite standard calibration curve (0–100 µM) is performed for nitrite concentration estimation.

3.3.2 Fluorometric Detection of NO Production

1. Add DAF-FM diacetate(10 µM, Molecular Probes) to the culture medium and incubate in the dark for 20 min prior measurement (*see* **Note 12**).
2. Measure NO fluorescence intensity (excitation 495 nm; emission 515 nm) using a fluorescence plate reader or visualize NO dependent green fluorescence under an inverted fluorescence microscope. A culture without NOS protein as negative control is essential, with the same optical density (*see* **Note 12**).

4 Notes

1. The DNA sequence can be obtained from RNA extracted from the organism and preparing cDNA with transcriptase reverse and DNA by PCR or DNA synthesized commercially from genomic sequence databases [5]. The pET expression system (Novagen) is one of the most used systems for the cloning and in vivo expression of recombinant proteins in *E. coli*. This is due to the high selectivity of the pET system's bacteriophage T7 RNA polymerase for its cognate promoter sequences, the high level of activity of the polymerase and the high translation efficiency mediated by the T7 gene *10* translation initiation signals. In the pET system, the protein coding sequence of interest is cloned downstream of the T7 promoter and gene *10* leader sequences, and then transformed into *E. coli* strains. Protein expression is achieved by IPTG induction. Due to the specificity of the T7 promoter, basal expression of cloned target genes is extremely low in strains lacking a source of T7 RNA polymerase. This phenomenon, together with high-efficiency translation, achieves expression levels in which the target protein may constitute the majority of the cellular protein after only a few hours [12].
2. The pET24b-OtNOS vector is used to transform BL21 (DE3) protease-deficient *E. coli* via electroporation [5]. The BL21 (DE3) competent cells are an all purpose strain for high-level protein expression and easy induction. This strain is deficient in the proteases ompT and lon, resulting in a suitable host for recombinant expression [13].
3. *E.coli* is the most frequently used host for production of NOS enzymes [6, 14, 15]. Only mammalian iNOS requires of CaM co-expression to generate a fully active form expressed in *E. coli* [16].
4. The IPTG concentration used for NOS expression depends on the protein and the vector used for the expression system; it can vary between 0.1 and 2 mM. The temperature and time of induction depends on the NOS protein. The optimal conditions should be tested for each protein.
5. OtNOS expression can be identified by specific anti-OtNOS antibody [5].
6. The activities of the three NOS recombinant isoforms from animals are found distributed between the soluble and particulate fractions of cells. Isoform I (nNOS from brain) and isoform II (iNOS from cytokine-induced macrophages) are mostly soluble proteins. Isoform III (eNOS) from endothelial cells is myristoylated and found predominantly in the particulate fraction. The activities of isoforms I and III are regulated

by Ca^{2+} in the nanomolar range. The activity of isoform II is Ca^{2+} independent. Similar, but not identical procedures are used to purify the different isozymes. All three isoforms are hemoproteins and require the same cofactors, NADPH (6*R*)-5,6,7,8-BH_4, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN). Flavins and BH_4 are found bound to the purified enzymes in quantities that are sometimes, but not always, sufficient for full activity [17].

7. The OtNOS and eNOS recombinant proteins were purified using 2′5′-ADP Sepharose 4B and appeared as a single band of apparent molecular mass 119 and 135 kDa, respectively, on SDS/PAGE [5, 14]. 2′5′ADP Sepharose 4B media with immobilized NADPH structural analog is primarily used for purification of enzymes requiring NADPH as a cofactor.

   The addition of a specific tag (e.g., His tag) is a common strategy used to express and purify NOS proteins. NOS cDNA sequences from *Bacillus subtilis* and *Deinococcus radiodurans* were cloned with a His_6 tag attached to its N terminus and the proteins were over expressed in *E. coli* strain BL21 (DE3) and purified using chromatography on Ni^{2+}-nitrilotriacetic acid resin [18, 19].

8. Spectrophotometry has been widely used to measure NOS activity through the oxyhemoglobin (oxyHb) assay. NO stoichiometrically reacts with oxyHb to produce methemoglobin (metHb) [20]. The distinct optical behaviors of these species (oxyHb and metHb) make the conversion of oxyHb to metHb a simple, practical a rapid spectrophotometric determination of NOS activity [20]. Nevertheless; same precautions have to be taken when using this assay. The isolated NOS protein should be preferably not contaminated with other proteins, normally a minimal metHb formation is observed without proteins in the sample. Hence, it is necessary the control reaction performed either (a) with a specific inhibitor of NOS, (b) in absence of the cofactor nicotinamide adenine dinucleotide phosphate (NADPH) or (c) without the substrate l-Arg. This assay is commonly used for the analysis of the catalytic properties of NOS [5, 19–21].

9. NOS isoforms catalyze other leak and side reactions, such as superoxide production at the expense of NADPH. As such, this stoichiometry is not generally observed, and reflects the three electrons supplied by NADPH to form one NO. A partial uncouple reaction in the NOS activity can be detected by NADPH oxidation [22].

10. The l-citrulline assay is based on the stoichiometric production of NO and l-[^{3}H]-citrulline from l-[^{3}H]-arginine by NOS. This chemical reaction is the basis of the l-citrulline

assay, a simple and specific method that is currently used to measure NOS enzymatic activity, with control reactions (blanks) performed either (a) with a specific inhibitor of NOS, (b) in absence of the cofactor NADPH, or (c) with a protein extract that has been boiled prior to the incubation. Since the reaction can be performed with or without Ca^{2+}, it can be discriminated between Ca^{2+}-dependent and Ca^{2+}-independent NOS activities. The l-citrulline assay has been widely used to demonstrate NOS activity in a variety of cells and tissues [19, 23, 24]. However, NOS activity is relatively unstable, and the l-citrulline assay is technically inapplicable when applied with low levels of NOS protein [25].

The principle of the technique is to retain the labeled l-[^{3}H]-arginine, by the Dowex and measured in the scintillation system. There are many reports of an arginine-dependent NOS activity in higher plants. To NOS activity in plants extracts, many studies have relied on a citrulline-based assay that measures the formation of l-citrulline from l-Arg using ion exchange chromatography. However, Tischner et al. [26] reported that when such assays were used with protein extracts from *Arabidopsis*, an l-Arg-dependent NOS activity was observed, but it generated a metabolite product other than l-citrulline. Indeed, TLC analysis identified the product as argininosuccinate. The reaction was stimulated by fumarate (>500 μM), suggesting that the enzyme involved was the urea cycle enzyme argininosuccinate lyase (EC 4.3.2.1), which reversibly converts l-Arg and fumarate to argininosuccinate. These results indicate that caution is needed when using standard citrulline-based assays to measure NOS activity in plant extracts, and highlight the importance of verifying the identity of the product as l-citrulline.

11. NO has a short half-life (<10 s), which makes it difficult to detect and study. However, as NO is metabolized to nitrate and nitrite in the cell, quantization of these stable anions can be used to measure the amount of NO that was originally present in a sample [26]. The Griess reaction was reported by Johann Peter Griess in 1879 as a method of analysis of nitrite. In this method, nitrite is first treated with a diazotizing reagent (Reagent A), e.g., sulfanilamide (SA), in acidic media to form a transient diazonium salt. This intermediate is then allowed to react with a coupling Reagent B, N-naphthylethylenediamine (NED), to form a stable azo compound. The intense purple color of the product allows assaying nitrite with high sensitivity and can be used to measure nitrite concentration as low as ~0.5 μM. The absorbance at 540 nm of the formed adduct is linearly proportional to the nitrite concentration in the sample [27]. Through the years, many variations

on the original reaction have been described. The most popular version seems to be the sequential method in which nitrite is mixed with SA first, followed by the addition of NED. This method seems to give highest yield of the chromophore, and therefore it is the most sensitive way to perform Griess Reaction assay [28, 29]. For a more accurate measurement of NO produced in a sample, the nitrate formed via oxidation of nitrite must also be measured. This is often accomplished by reducing nitrate to nitrite immediately prior to the addition of the Griess reagents to the initial sample [30]. This approach was employed to identify NOS activity in *E. coli* expressing a recombinant NOS, including OtNOS and NOS from *Bacillus subtilis* [5, 6]. The NOS activity in vitro can be measured by Griess reagent, however, NADPH, an essential cofactor for NOS activity, interferes with the Griess reaction, which severely limits the sensitivity of conventional assays. The addition of lactate dehydrogenase (LDH) eliminates NADPH and can increase the sensitivity of the Griess reaction.

Some considerations and recommendations for the Griess assay:

- Sulfanilamide and NED compete for nitrite in the Griess reaction [30], and thus greater sensitivity is achieved when the two components are added sequentially.
- The final pH of a sample after addition of reagent A is critical for the Griess reaction. Lower final pH results in higher absorbance at 540 nm. When the pH is lower than 1.8, the absorbance at 540 nm is stable [27]. For samples with a high buffer capacity more acid should be added to reagent A.
- In a condition where there is low level of NO production, high amount of nitrate (or nitrite) in the media will make the measurement difficult due to the high background [30], and thus it is important to know minutely nitrite and nitrate concentration in the media broth used and also to minimize as much as possible the content of these compounds from water in the solutions.

12. DAF-FM (4-aminomethyl-5-methylamino-2′,7′-difluorofluorescein) and DAF-FM diacetate represent two important reagent for quantification of low concentrations of NO (~5 nM). Developed by Kojima et al. [33], these compounds are essentially nonfluorescent until they react with NO to form a fluorescent benzotriazole. DAF-FM diacetate is cell-permeable and passively diffuses across cellular membranes. Once inside cells, it is deacetylated by intracellular esterases to become DAF-FM. With excitation/emission maxima of 495/515 nm, DAF-FM can be detected by any instrument that can detect fluorescein, including flow cytometers, microscopes, fluorescent

microplate readers, and fluorometers. Probably the most successful indicator for nitric oxide has been 4,5-diaminofluorescein diacetate (DAF-2 diacetate), which was also developed by Kojima and collaborators [33, 34]. DAF-2 has been used to identify NO-production in plant cells [33]. The DAF-FM reagent has some important advantages over DAF-2. The spectra of the NO adduct of DAF-FM are independent of pH above pH 5.5. Also, the NO adduct of DAF-FM is significantly more photo-stable than that of DAF-2 which means additional time for image capture. Finally, DAF-FM is a more sensitive reagent for NO than is DAF-2 (NO detection limit for DAF-FM ~3 nM vs. ~5 nM for DAF-2) [32–34]. DAF-FM diacetate was used for NOS activity detection in *E. coli* expressing OtNOS [5]. Gusarov et al. [6] utilized the NO-specific fluorescent probe CuFL, which has only recently become available [35], to directly monitor NO production by bNOS expressed in *E. coli*. A cell culture without NOS protein as negative control is essential within a current experimental protocol.

References

1. Muller U (1997) The nitric oxide system in insects. Prog Neurobiol 51(3):363–381
2. Klessig DF, Durner J, Noad R, Navarre DA, Wendehenne D, Kumar D, Zhou JM, Shah J, Zhang S, Kachroo P, Trifa Y, Pontier D, Lam E, Silva H (2000) Nitric oxide and salicylic acid signaling in plant defense. Proc Natl Acad Sci U S A 97(16):8849–8855
3. Golderer G, Werner ER, Leitner S, Grobner P, Werner-Felmayer G (2001) Nitric oxide synthase is induced in sporulation of *Physarum polycephalum*. Genes Dev 15(10):1299–1309. doi:10.1101/gad.890501
4. Agapie T, Suseno S, Woodward JJ, Stoll S, Britt RD, Marletta MA (2009) NO formation by a catalytically self-sufficient bacterial nitric oxide synthase from *Sorangium cellulosum*. Proc Natl Acad Sci U S A 106(38):16221–16226. doi:10.1073/pnas.0908443106
5. Foresi N, Correa-Aragunde N, Parisi G, Calo G, Salerno G, Lamattina L (2010) Characterization of a nitric oxide synthase from the plant kingdom: NO generation from the green alga *Ostreococcus tauri* is light irradiance and growth phase dependent. Plant Cell 22(11):3816–3830. doi:10.1105/tpc.109.073510
6. Gusarov I, Starodubtseva M, Wang ZQ, McQuade L, Lippard SJ, Stuehr DJ, Nudler E (2008) Bacterial nitric-oxide synthases operate without a dedicated redox partner. J Biol Chem 283(19):13140–13147. doi:10.1074/jbc.M710178200
7. Andreakis N, D'Aniello S, Albalat R, Patti FP, Garcia-Fernàndez J, Procaccini G, Sordino P, Palumbo A (2011) Evolution of the nitric oxide synthase family in metazoans. Mol Biol Evol 28(1):163–179. doi:10.1093/molbev/msq179
8. Alderton WK, Cooper CE, Knowles RG (2001) Nitric oxide synthases: structure, function and inhibition. Biochem J 357(Pt 3):593–615
9. Salerno JC, Harris DE, Irizarry K, Patel B, Morales AJ, Smith SM, Martasek P, Roman LJ, Masters BS, Jones CL, Weissman BA, Lane P, Liu Q, Gross SS (1997) An autoinhibitory control element defines calcium-regulated isoforms of nitric oxide synthase. J Biol Chem 272(47):29769–29777
10. Wever RM, van Dam T, van Rijn HJ, de Groot F, Rabelink TJ (1997) Tetrahydrobiopterin regulates superoxide and nitric oxide generation by recombinant endothelial nitric oxide synthase. Biochem Biophys Res Commun 237(2):340–344. doi:10.1006/bbrc.1997.7069
11. Derelle E, Ferraz C, Rombauts S, Rouze P, Worden AZ, Robbens S, Partensky F, Degroeve S, Echeynie S, Cooke R, Saeys Y, Wuyts J, Jabbari K, Bowler C, Panaud O, Piegu B, Ball SG, Ral JP, Bouget FY, Piganeau G, De Baets B, Picard A, Delseny M, Demaille J, Van de Peer Y, Moreau H (2006) Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features. Proc Natl Acad Sci U S A 103(31):11647–11652. doi:10.1073/pnas.0604795103

12. Studier FW (1991) Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. J Mol Biol 219(1):37–44
13. Sorensen HP, Mortensen KK (2005) Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. Microb Cell Factories 4(1):1. doi:10.1186/1475-2859-4-1
14. Martasek P, Liu Q, Liu J, Roman LJ, Gross SS, Sessa WC, Masters BS (1996) Characterization of bovine endothelial nitric oxide synthase expressed in *E. coli*. Biochem Biophys Res Commun 219(2):359–365. doi:10.1006/bbrc.1996.0238
15. Fossetta JD, Niu XD, Lunn CA, Zavodny PJ, Narula SK, Lundell D (1996) Expression of human inducible nitric oxide synthase in *Escherichia coli*. FEBS Lett 379(2):135–138
16. Wu C, Zhang J, Abu-Soud H, Ghosh DK, Stuehr DJ (1996) High-level expression of mouse inducible nitric oxide synthase in Escherichia coli requires coexpression with calmodulin. Biochem Biophys Res Commun 222(2):439–444. doi:10.1006/bbrc.1996.0763
17. Forstermann U, Closs EI, Pollock JS, Nakane M, Schwarz P, Gath I, Kleinert H (1994) Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. Hypertension 23(6 Pt 2):1121–1131
18. Adak S, Aulak KS, Stuehr DJ (2002) Direct evidence for nitric oxide production by a nitric-oxide synthase-like protein from *Bacillus subtilis*. J Biol Chem 277(18):16167–16171. doi:10.1074/jbc.M201136200
19. Wang ZQ, Lawson RJ, Buddha MR, Wei CC, Crane BR, Munro AW, Stuehr DJ (2007) Bacterial flavodoxins support nitric oxide production by *Bacillus subtilis* nitric-oxide synthase. J Biol Chem 282(4):2196–2202. doi:10.1074/jbc.M608206200
20. Ghafourifar P, Asbury ML, Joshi SS, Kincaid ED (2005) Determination of mitochondrial nitric oxide synthase activity. Methods Enzymol 396:424–444. doi:10.1016/S0076-6879(05)96036-9
21. Rodriguez-Crespo I, Ortiz de Montellano PR (1996) Human endothelial nitric oxide synthase: expression in *Escherichia coli*, coexpression with calmodulin, and characterization. Arch Biochem Biophys 336(1):151–156
22. Griffith OW, Stuehr DJ (1995) Nitric oxide synthases: properties and catalytic mechanism. Annu Rev Physiol 57:707–736. doi:10.1146/annurev.ph.57.030195.003423
23. Knowles RG, Salter M (1998) Measurement of NOS activity by conversion of radiolabeled arginine to citrulline using ion-exchange separation. Methods Mol Biol 100:67–73
24. Ward TR, Mundy WR (1999) Measurement of the nitric oxide synthase activity using the citrulline assay. Methods Mol Med 22:157–162. doi:10.1385/0-89603-612-X:157
25. Combet S, Balligand JL, Lameire N, Goffin E, Devuyst O (2000) A specific method for measurement of nitric oxide synthase enzymatic activity in peritoneal biopsies. Kidney Int 57(1):332–338. doi:10.1046/j.1523-1755.2000.00839.x
26. Tischner R, Galli M, Heimer YM, Bielefeld S, Okamoto M, Mack A, Crawford NM (2007) Interference with the citrulline-based nitric oxide synthase assay by argininosuccinate lyase activity in Arabidopsis extracts. FEBS J 274(16):4238–4245. doi:10.1111/j.1742-4658.2007.05950.x
27. Xu J, Xu X, Verstraete W (2000) Adaptation of E. coli cell method for micro-scale nitrate measurement with the Griess reaction in culture media. J Microbiol Methods 41(1):23–33
28. Verdon CP, Burton BA, Prior RL (1995) Sample pretreatment with nitrate reductase and glucose-6-phosphate dehydrogenase quantitatively reduces nitrate while avoiding interference by NADP+ when the Griess reaction is used to assay for nitrite. Anal Biochem 224(2):502–508. doi:10.1006/abio.1995.1079
29. Guevara I, Iwanejko J, Dembinska-Kiec A, Pankiewicz J, Wanat A, Anna P, Golabek I, Bartus S, Malczewska-Malec M, Szczudlik A (1998) Determination of nitrite/nitrate in human biological material by the simple Griess reaction. Clin Chim Acta 274(2):177–188
30. Arita NO, Cohen MF, Tokuda G et al (2007) Fluorometric detection of nitric oxide with diaminofluoresceins (DAFs): applications and limitations for plant NO research. In: Polaco JC, Lamattina L (eds) Nitric oxide in plant growth, development and stress physiology. Springer, Berlin, pp 269–280
31. Fiddler RN (1977) Collaborative study of modified AOAC method of analysis for nitrite in meat and meat products. J Assoc Off Anal Chem 60(3):594–599
32. Foissner I, Wendehenne D, Langebartels C, Durner J (2000) In vivo imaging of an elicitor-induced nitric oxide burst in tobacco. Plant J 23(6):817–824
33. Kojima H, Nakatsubo N, Kikuchi K, Kawahara S, Kirino Y, Nagoshi H, Hirata Y, Nagano T (1998) Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. Anal Chem 70(13):2446–2453
34. Kojima H, Urano Y, Kikuchi K, Higuchi T, Hirata Y, Nagano T (1999) Fluorescent indicators for imaging nitric oxide production. Angew Chem 38(21):3209–3212
35. Lim MH, Lippard SJ (2006) Fluorescent nitric oxide detection by copper complexes bearing anthracenyl and dansyl fluorophore ligands. Inorg Chem 45(22):8980–8989. doi:10.1021/ic0609913