

***In vitro* Nitrate Reductase Activity Assay of *Mycobacterium smegmatis* Crude Extract**

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[Abstract] Nitrate is one of the major inorganic nitrogen sources for microorganisms. Many bacterial and archaeal lineages can express cytoplasmic assimilatory nitrate reductase (NAS), which catalyzes the rate-limiting reduction of nitrate to nitrite in the nitrate assimilation pathway. Here, we present a detailed protocol for measuring *in vitro* nitrate reductase (NaR) activity of NAS enzymes from *Mycobacterium smegmatis* crude extract using both physiological and non-physiological electron donors.

Keywords: *Mycobacterium smegmatis*, Assimilatory nitrate reductase, *In vitro* nitrate reductase activity assay, NADPH, NADH, Methyl viologen

[Background] In addition to being an important nitrogen source for plants, nitrate (NO₃⁻) is universally used as an inorganic nitrogen source for microorganisms, particularly soil bacteria such as actinomycetes, as well as marine bacteria such as cyanobacteria, due to fierce competition for limited nutrients in the natural environment (Kuypers *et al.*, 2018). In prokaryotes, nitrate is reduced to ammonia (NH₃) through two sequential steps, with the first committed step catalyzed by NAS. Prokaryotic NASs are categorized based on the physiological electron donors utilized (Morozkina and Zvyagilskaya, 2007; Tan *et al.*, 2020), *i.e.*, the flavodoxin- or ferredoxin-dependent NAS and the NAD(P)H-dependent NAS; however, to date, there have been few reports detecting *in vitro* NaR activity of bacterial NAD(P)H-dependent NASs employing their physiological electron donors. Instead, methyl viologen (an artificial chemical reductant) is the most commonly used electron donor for *in vitro* NaR activity assays.

In this protocol, employing an optimized assay system, NAD(P)H-dependent NaR activity can be measured in *M. smegmatis* crude cell extract. Briefly, the enzyme samples prepared under anaerobic conditions can use both NADPH and NADH as electron donors for catalysis *in vitro*. Nitrite produced by nitrate reduction forms a colored azo compound with sulfanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride, which can be measured by its absorbance at 540 nm. This method is suitable for the determination of *in vitro* NaR activity of bacterial NAS enzymes.

Materials and Reagents

1. *Mycobacterium smegmatis* mc²155
2. Middlebrook 7H10 agar (BD Biosciences, catalog number: 262710)
3. Middlebrook 7H9 broth (BD Biosciences, catalog number: 271310)
4. Glycerol (Sigma-Aldrich, catalog number: G5516)
5. Tween 80 (Sigma-Aldrich, catalog number: P1754)
6. Ethanol (Sigma-Aldrich, catalog number: 51976)
7. Phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: P7626)
8. Bradford protein assay kit (Beyotime, catalog number: P0006)
9. Sodium nitrate (NaNO₃) (Sigma-Aldrich, catalog number: S5506)
10. Sodium nitrite (NaNO₂) (Sigma-Aldrich, catalog number: S2252)
11. Sodium phosphate dibasic (Na₂HPO₄) (Sigma-Aldrich, catalog number: 255793)
12. Sodium phosphate monobasic (NaH₂PO₄) (Sigma-Aldrich, catalog number: S3139)
13. Nicotinamide adenine dinucleotide phosphate (NADPH) (Sigma-Aldrich, catalog number: N7505)
14. Nicotinamide adenine dinucleotide (NADH) (Sigma-Aldrich, catalog number: 43420)
15. Methyl viologen (Sigma-Aldrich, catalog number: 856177)
16. Flavin mononucleotide (FMN) (Sigma-Aldrich, catalog number: F2253)
17. Flavin adenine dinucleotide (FAD) (Sigma-Aldrich, catalog number: F6625)
18. Sodium hydrosulfite (Na₂S₂O₄) (Sigma-Aldrich, catalog number: 71699)
19. Sodium bicarbonate (NaHCO₃) (Sigma-Aldrich, catalog number: S6014)
20. Sulfanilamide (Sigma-Aldrich, catalog number: S9251)
21. N-(1-naphthyl)ethylenediamine dihydrochloride (NED) (Sigma-Aldrich, catalog number: 222488)
22. 7H10 agar (see Recipes)
23. 7H9 medium (see Recipes)
24. MPLN medium (see Recipes)
25. 0.5 M sodium phosphate buffer, pH 7.5 (see Recipes)
26. 0.1 M sodium phosphate buffer, pH 7.5 (see Recipes)
27. 0.1 M PMSF (see Recipes)
28. 60 mM Na₂S₂O₄/NaHCO₃ (see Recipes)
29. 1% (w/v) sulfanilamide solution (see Recipes)
30. 0.01% (w/v) NED solution (see Recipes)

Equipment

1. Pipette kit (Gilson, catalog number: F167300)
2. High-speed centrifuge (Beckman, catalog number: AVANTI JXN-26)
3. [Ultrasonic cell disruptor](#) (Beyotime, catalog number: E0385)
4. Ultrasonic bath equipped with a degas mode (Beyotime, catalog number: E0439)

5. 250-ml and 2-L Erlenmeyer flasks
6. Petri dish (90 mm diameter)
7. 37°C incubator
8. Incubator shaker
9. Water bath
10. Spectrophotometer
11. Anaerobic chamber (glove box) filled with mixed gas (nitrogen, 95%; hydrogen, 5%)

Note: Place the pipette kit, [ultrasonic cell disruptor](#), and water bath inside the anaerobic chamber.

Software

1. Microsoft Excel

Procedure

A. Preparation of *M. smegmatis* culture

1. Streak *M. smegmatis* frozen culture on a 7H10 agar plate and incubate aerobically at 37°C for 3 days until colonies are visible.
2. Inoculate a single colony into 50 ml 7H9 medium in a 250-ml Erlenmeyer flask and incubate aerobically at 37°C for 16 h with shaking at 180 rpm (overnight culture).
3. Collect cells from the overnight culture by centrifuging at $3,000 \times g$ for 10 min. Wash the pellet twice with nitrogen-free MPLN medium and centrifuge as above. Resuspend the cells in 50 ml MPLN medium as the seeding culture.
4. Re-inoculate 10 ml seeding culture into 1 L MPLN medium supplemented with 10 mM nitrate in a 2-L Erlenmeyer flask and incubate aerobically at 37°C for 30 h with shaking to late-log phase (OD_{600} 0.8–1.0).

B. Preparation of *M. smegmatis* crude extract

1. Collect cells by centrifuging at $5,000 \times g$ for 10 min at 4°C.
2. Wash the cell pellet twice with 30 ml degassed 0.1 M sodium phosphate buffer (pH 7.5).
3. Transfer the cell pellet into an anaerobic chamber and resuspend in 20 ml degassed 0.1 M sodium phosphate buffer (pH 7.5) supplemented with 10% v/v glycerol and 1 mM PMSF.
4. Disrupt cells by sonication (pulse: 0.3 s, stop: 0.7 s) for 10 min on ice and transfer the lysate to a centrifuge tube inside the anaerobic chamber.
5. Centrifuge at $16,000 \times g$ for 1 h at 4°C outside the anaerobic chamber.
6. Bring the centrifuge tube back into the anaerobic chamber and collect the supernatant (cytoplasmic fraction) as the crude enzyme extract.

Note: Keep on ice during the assay and use within 24 h.

7. Determine the total protein concentration of the cytoplasmic fraction using the Bradford method.

C. Preparation of the nitrite standard curve

1. Prepare a nitrite dilution series using sodium nitrite (0, 3, 30, 60, 120, 240, and 300 μM) in deionized water.
2. Pipette 0.5 ml nitrite dilution series (Test sample) into 1.5-ml tubes. Use 0.5 ml 10 mM sodium nitrate as a control (Blank sample).
3. Add 0.5 ml sulfanilamide solution and 0.5 ml NED solution to all tubes.
4. Mix by swirling and incubate at 25°C for 10 min.
5. Transfer 1 ml assay mixture to a cuvette.
6. Measure the visible absorption at 540 nm using the spectrophotometer.
7. Calculate the absorbance change using the following equation:
$$\Delta A_{540 \text{ nm}} (\text{Sample}) = A_{540 \text{ nm}} (\text{Test}) - A_{540 \text{ nm}} (\text{Blank})$$
8. Plot the standard curve with the nitrite concentration on the horizontal axis and the absorbance change on the vertical axis (Figure 1).

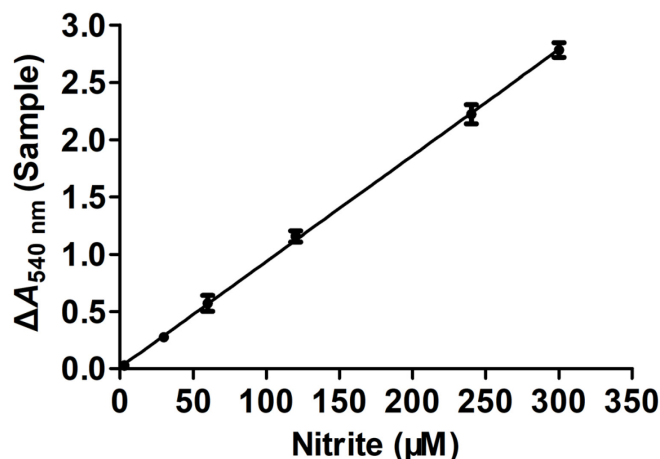


Figure 1. Nitrite standard curve. Error bars indicate the average value of three biological replicates $\pm$ SD ($n = 3$). According to the standard curve, the regression equation is $x (\mu\text{M}) = (y - 0.0204)/0.0092$, where x stands for nitrite concentration and y stands for $\Delta A_{540 \text{ nm}} (\text{Sample})$.

D. *In vitro* NaR activity assay

1. Before preparing the reaction mixture for the enzyme assay, degas the solutions used in the assays and place in the anaerobic chamber before use.
2. Pipette the following reagents for the nitrate reduction reaction (Tables 1 and 2) into a 1.5-ml tube inside the anaerobic chamber:

Table 1. Assay mixtures for determining NAD(P)H-dependent NaR activity

	Assay sample	
	Test	Blank

Deionized water	0.090 ml	0.100 ml
Sodium phosphate buffer (0.5 M)	0.100 ml	0.100 ml
NaNO ₃ (50 mM)	0.100 ml	0.100 ml
NADPH (2 mM) or NADH (2 mM)	0.100 ml	0.100 ml
FMN (0.25 mM)	0.050 ml	0.050 ml
FAD (0.25 mM)	0.050 ml	0.050 ml
Crude enzyme extract	0.010 ml	-
Step 1: Mix gently and incubate at 30°C for 10 min inside the anaerobic chamber		
1% (w/v) sulfanilamide solution	0.500 ml	0.500 ml
0.01% (w/v) NED solution	0.500 ml	0.500 ml
Step 2: Mix gently and incubate at 25°C in the dark for 10 min inside the anaerobic chamber		

Table 2. Assay mixtures for determining methyl viologen-dependent NaR activity

	Assay sample	
	Test	Blank
Deionized water	0.090 ml	0.100 ml
Sodium phosphate buffer (0.5 M)	0.100 ml	0.100 ml
NaNO ₃ (50 mM)	0.100 ml	0.100 ml
Methyl viologen (0.75 mM)	0.100 ml	0.100 ml
Na ₂ S ₂ O ₄ /NaHCO ₃ (60 mM)	0.100 ml	0.100 ml
Crude enzyme extract	0.010 ml	-
Step 1: Mix gently and incubate at 30°C for 10 min inside the anaerobic chamber		
Step 2: Stop the reaction by vigorous stirring in the open air outside the anaerobic chamber until the blue color disappears		
1% (w/v) sulfanilamide solution	0.500 ml	0.500 ml
0.01% (w/v) NED solution	0.500 ml	0.500 ml
Step 3: Mix gently and incubate at 25°C in the dark for 10 min outside the anaerobic chamber		

Note: The assay mixture turns deep blue after adding Na₂S₂O₄/NaHCO₃, an indication of methyl viologen being reduced.

- Transfer 1 ml assay mixture to a cuvette.
- Measure the visible absorption at 540 nm for both the Test and Blank samples.
- Calculate the NaR specific activity using the following equation:

$$\text{NaR specific activity (nmol/min/mg)} = C_{\text{nitrite}} \text{ DF} / T / C_{\text{enzyme}} / V_{\text{enzyme}}$$

where,

$C_{\text{nitrite}} (\mu\text{M}) = [A_{540 \text{ nm}} (\text{Test}) - A_{540 \text{ nm}} (\text{Blank}) - 0.0204] / 0.0092$, the concentration of nitrite formed in the assay

DF: The dilution factor

T (min): The reaction time for nitrate reduction in the assay

Note: An incubation time of 10 min is sufficient for measuring the in vitro nitrate reductase activity.

$C_{\text{enzyme}} (\text{mg/ml})$: The protein concentration of the crude enzyme extract

$V_{\text{enzyme}} (\text{ml})$: The volume of the crude enzyme extract used in the assay

Recipes

1. 7H10 agar

Dissolve 19 g 7H10 powder in 1 L deionized water

Add 4 ml 50% (v/v) glycerol and 5 ml 10% (v/v) Tween 80

Autoclave at 121°C for 15 min

2. 7H9 medium

Dissolve 4.7 g 7H9 powder in 1 L deionized water

Add 4 ml 50% (v/v) glycerol and 5 ml 10% (v/v) Tween 80

Autoclave at 121°C for 15 min

3. Nitrogen-free MPLN medium (pH 6.6)

36.7 mM KH_2PO_4

7.7 mM sodium citrate

5 mM MgSO_4

60 μM FeCl_3

20 μM NaMoO_4

3.4 μM CaCl_2

3.5 μM ZnSO_4

4 μM CuSO_4

2% (v/v) glycerol

0.05% (v/v) Tween 80

Autoclave at 121°C for 15 min.

4. 0.5 M sodium phosphate buffer (pH 7.50)

Prepare 0.5 M solutions of NaH_2PO_4 and Na_2HPO_4

Mix 20% (v/v) NaH_2PO_4 and 80% (v/v) Na_2HPO_4

Adjust pH to 7.5

5. 0.1 M sodium phosphate buffer (pH 7.50)

Dilute 0.5 M sodium phosphate buffer 1:5 with deionized water

Store at room temperature

6. 0.1 M PMSF

Dissolve 0.174 g PMSF in 10 ml ethanol

Store at -20°C

7. 60 mM Na₂S₂O₄/NaHCO₃

Prepare 120 mM solutions of Na₂S₂O₄ and NaHCO₃

Mix 50% (v/v) Na₂S₂O₄ and 50% (v/v) NaHCO₃

Note: Prepare fresh

8. 1% (w/v) sulfanilamide solution

Dissolve in 3 M HCl

Note: Prepare fresh and store at 4°C in the dark

9. 0.01% (w/v) NED solution

Dissolve in deionized water

Note: Prepare fresh and store at 4°C in the dark

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Competing interests

The authors declare that they have no conflicts of interest.

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