Enzymatic Assay via NAD(P)H Monitoring for Human and Microbial Steroid 5β-Reductases

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Materials

- Purified enzyme preparation (record protein concentration, purification date, and storage conditions).
- NADH or NADPH, depending on the enzyme being investigated.
- Testosterone stock solution (40 mM in absolute ethanol).
- Potassium phosphate buffer (100 mM, pH 6.5).
- UV-transparent microcuvettes suitable for absorbance measurements at 340 nm.
- UV–visible spectrophotometer capable of wavelength scans and kinetic measurements.
- Ice and aluminium foil for cofactor storage during the experiment.

Testosterone Stock Solution

Testosterone — Sigma-Aldrich **For authorized personnel only.**

Prepare testosterone stock solution according to the approved laboratory procedure. Store in appropriately labelled aliquots and minimize solvent evaporation, which could alter the effective testosterone concentration.

Starting Reaction Conditions

The reaction is adapted to a final volume of 1 mL in a UV-transparent microcuvette.

Component	Working stock	Volume	Final concentration
Potassium phosphate buffer	100 mM, pH 6.5	929.75 µL	—
NADPH or NADH	5 mM	20 µL	100 µM

Testosterone in absolute ethanol	40 mM	0.25 µL	10 µM
Enzyme preparation		50 µL	
Total volume		**1,000 µL**	

Important notes:

- Use NADPH or NADH according to the enzyme being tested. Do not assume that the cofactors are interchangeable.
- Direct pipetting of 0.25 µL testosterone stock is not recommended. Prepare a suitable working dilution or a reaction master mix to improve pipetting accuracy.
- High cofactor concentrations are meant to not limit the reaction

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Experimental Layout and Controls

Perform measurements in duplicate or triplicate, preferably using independently prepared reaction mixtures.

Condition	Testosterone	Cofactor	Enzyme preparation
Complete reaction	Yes	Yes	Yes
No-substrate control	Ethanol only	Yes	Yes
No-enzyme control	Yes	Yes	Enzyme-storage buffer
No-cofactor background	Yes	No	Yes
Empty-vector control, if available	Yes	Yes	Purified empty-vector preparation

For every reaction:

- Maintain a final volume of 1 mL.
- Maintain an identical final ethanol concentration across matched conditions.
- Replace omitted components with the appropriate buffer or solvent.
- Use the same enzyme-preparation volume in comparable reactions.
- Record the enzyme concentration, cofactor identity, and final reaction composition.

The no-substrate control evaluates cofactor consumption in the absence of added testosterone. The no-enzyme control evaluates the background behavior of the reaction components. Empty-vector preparations can help identify signals associated with host-derived proteins or the purification workflow.

Spectrophotometer Settings

Parameter	Setting
Measurement wavelength	340 nm
Temperature	Room temperature
Measurement interval	Every 20–30 seconds
Monitoring duration	At least 30 minutes
Measurement mode	Absorbance kinetics
Cuvette	UV-transparent, suitable for 1 mL reactions

Step-by-Step Procedure

1. Spectrophotometer Preparation

1. Turn on the spectrophotometer and select the appropriate wavelength-scan or kinetic measurement program.
2. Prepare a reduced-cofactor solution at a concentration appropriate for detecting its characteristic absorbance peak near 340 nm.
3. Acquire a wavelength scan to confirm the presence of the expected NADH or NADPH absorbance peak.
4. Prepare a suitable blank containing the buffer and relevant solvent components, without the reduced cofactor.

2. Reagent Preparation

6. Prepare fresh NADH or NADPH working solutions. Protect it from direct light and keep it on ice during the experiment.
7. Check that the testosterone stock and working solutions are free of visible precipitate.
8. Prepare the potassium phosphate buffer and verify its pH.
9. Keep enzyme preparations on ice until use.

3. Reaction Preparation

10. Prepare reaction master mixes according to the experimental conditions. Ensure that all reactions contain the same total volume and appropriately matched solvent concentrations.
11. Include the required negative controls.
12. Prepare sufficient master mix for all replicates, allowing a small excess for pipetting losses.
13. Equilibrate the assay components to the selected measurement temperature before recording kinetics.

4. Kinetic Measurements

14. Add 20 µL of the 5 mM NADH or NADPH working solution to the cuvette.
15. Add 980 µL of the appropriate reaction master mix, ensuring that the total reaction volume is 1 mL.
16. Mix carefully without introducing bubbles.
17. Immediately begin recording absorbance at 340 nm.
18. Record measurements every 20–30 seconds for at least 30 minutes.
19. Repeat the procedure for all reaction and control conditions.

Note: This addition order defines the beginning of the reaction as the mixing of cofactor with the enzyme-containing master mix. If you intend to initiate the reaction by adding the enzyme instead, the reaction setup must be adjusted consistently.