

Version 3a Last updated 3 April 2020

ab221821 NAD/NADH Assay Kit II (Colorimetric)

For the rapid, sensitive and accurate measurement of NAD/NADH in cell lysates.

[View kit datasheet: www.abcam.com/ab221821](http://www.abcam.com/ab221821)

(use www.abcam.cn/ab221821 for China, or www.abcam.co.jp/ab221821 for Japan)

This product is for research use only and is not intended for diagnostic use.

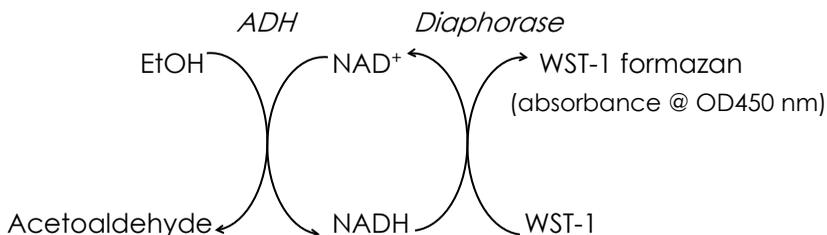
Table of Contents

1. Overview	1
2. Protocol Summary	2
3. Precautions	3
4. Storage and Stability	3
5. Limitations	4
6. Materials Supplied	4
7. Materials Required, Not Supplied	5
8. Technical Hints	6
9. Reagent Preparation	7
10. Standard Preparation	8
11. Sample Preparation	9
12. Assay Procedure	11
13. Calculations	13
14. Typical Data	15
15. Troubleshooting	20
16. Interferences	22
17. FAQs	22
18. Notes	23

1. Overview

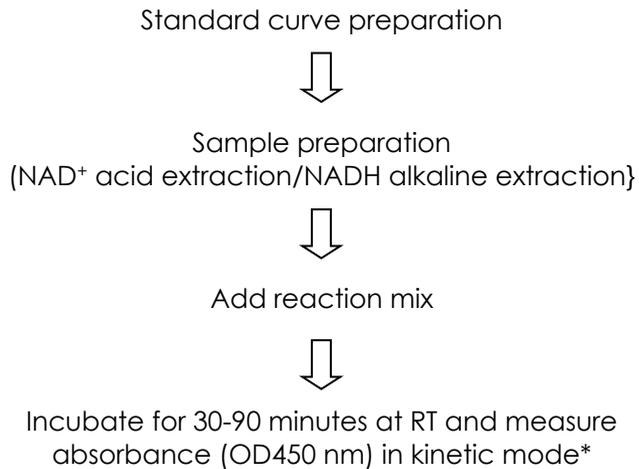
NAD/NADH Assay Kit II (Colorimetric) (ab2111821) provides a sensitive and robust method to measure NAD⁺, NADH and their ratio in mammalian cell lysates. The assay is based on an ADH and diaphorase coupled-reaction that converts WST-1 to WST-1 formazan, which can be easily detected at OD 450 nm. As the reaction is not stopped, it is necessary to monitor the absorbance increase of WST-1 formazan at regular intervals after the reaction is initiated to determine the reaction velocity.

This assay requires purification of NAD⁺ and NADH from the cell lysates, which raises the efficiency of the reaction and increases the detection sensitivity.



Nicotinamide nucleotides are key players in the energy and oxidation-reduction reactions of a cells. Nicotinamide adenine dinucleotide (NAD) exists in two forms, an oxidized form, NAD⁺, and a reduced form, NADH. NAD functions as a cofactor in the vast majority of cellular redox reactions, carrying reducing equivalents from one reaction to another. Therefore, maintaining appropriate levels of NAD is essential for maintaining normal cellular respiratory function. There are two major pathways in NAD biosynthesis. The *de novo* pathway is maintained by the rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT), whereas the salvage pathway recycles degraded NAD products such as nicotinamide. Studies have shown that cytosolic NAD⁺ concentrations range from 300 nM in mammalian cells to 2 mM in yeast. Depletion of NAD in cells is a major cause of cell death. The importance of NAD function in modulating cellular redox status and controlling signaling and transcriptional events makes NAD an important cofactor when investigating normal cellular function.

2. Protocol Summary



**For kinetic mode detection, incubation time given in this summary is for guidance only*

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at -80°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

Δ Note: Reconstituted components are stable for 2 months.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
20X NAD/NADH Assay Buffer	1 mL	-80°C	-20°C
20X Standard Dilution Buffer	1 mL	-80°C	-20°C/4°C
WST-1	0.5 mL	-80°C	-80°C
ADH	0.5 mL	-80°C	-80°C
Diaphorase	0.5 mL	-80°C	-80°C
EtOH solution	0.5 mL	-80°C	-80°C
NADH Standard (400 µM)	0.2 mL	-80°C	-80°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at OD 450 nm – ideally, microplate should be capable of measuring dual wavelengths at 450/540 nm (range 450/550-450/590 nm)
- Double distilled water (ddH₂O)
- PBS
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom
- (Optional) 1 N H₂SO₄ (Stop Solution)
- (Optional) FK866 (specific NAMPT inhibitor, which will cause gradual NAD⁺ depletion): we recommend ab142148 – prepare 0.4 mM stock solution in DMSO
- (Optional) BCA protein assay kit (reducing agent compatible): we recommend using BCA protein assay kit reducing agent compatible (microplate) (ab207003)

For preparing NAD⁺ and NADH extracts from cell lysates:

- NAD⁺ Extraction Solution: 0.5 M perchloric acid (HClO₄)
- NAD⁺ Neutralization Solution: 0.55 M K₂CO₃
- NADH Extraction Solution: 50 mM NaOH + 1 mM EDTA
- NADH Neutralization Solution: 0.3 M Potassium Phosphate Buffer (pH 7.4)

8. Technical Hints

- **This kit is sold based on number of tests. A “test” simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Samples generating values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 20X NAD/NADH Assay Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store at -80°C .

9.2 20X Standard Dilution Buffer:

Prepare a working solution of Standard Dilution Buffer by diluting 20X stock 1:20 in ddH₂O and mixing well. Store at 4°C for short term (two weeks). Equilibrate to room temperature before use.

Store undiluted 20X Standard Dilution Buffer at -20°C .

9.3 WST-1:

Ready to use as supplied. Thaw on ice before use. Aliquot so that you have enough volume to performed the desired number of assays. Store at -80°C .

9.4 ADH:

Ready to use as supplied. Thaw on ice before use. Aliquot so that you have enough volume to performed the desired number of assays. Avoid freeze/thaw cycles. Store at -80°C .

9.5 Diaphorase:

Ready to use as supplied. Thaw on ice before use. Aliquot so that you have enough volume to performed the desired number of assays. Avoid freeze/thaw cycles. Store at -80°C .

9.6 Ethanol solution:

Ready to use as supplied. Thaw on ice before use. Aliquot so that you have enough volume to performed the desired number of assays. Store at -80°C .

9.7 NADH Standard (400 μM):

Ready to use as supplied. Thaw on ice before use. Aliquot so that you have enough volume to performed the desired number of assays. Store at -80°C .

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- Since NAD^+ is converted to NADH in enzyme cycling reaction and relatively labile than NADH, only NADH is provided in this kit.

10.1 Using 400 μM NADH standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Sample to dilute (μL)	Volume standard in well (μL)	Standard Dilution Buffer (μL)	End concentration NADH in well (nM/well)
1	400 μM stock	10	190	20,000
2	Std #1	100	100	10,000
3	Std #2	100	100	5,000
4	Std #3	100	100	2,500
5	Std #4	100	100	1,250
6	Std #5	100	100	625
7	Std #6	100	100	312.5
8	Blank (none)	0	100	0

Each dilution has enough amount of standard to set up duplicate readings ($2 \times 5 \mu\text{L}$).

11. Sample Preparation

General sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you snap freeze your samples in liquid nitrogen upon extraction and store them immediately at -80°C . When you are ready to test your samples, thaw them on ice and proceed with the Sample Preparation step. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1 Cell lysates:

11.1.1 Prepare Extraction and Neutralization solutions (Section 7):

- NAD^+ Extraction Solution: 0.5 M perchloric acid (HClO_4)
- NAD^+ Neutralization Solution: 0.55 M K_2CO_3
- NADH Extraction Solution: 50 mM NaOH + 1 mM EDTA
- NADH Neutralization Solution: 0.3 M Potassium Phosphate Buffer (pH 7.4)

11.1.2 Harvest the number of cells necessary for each assay (initial recommendation: $1-5 \times 10^6$ cells).

Δ Note: If using cells that have been previously frozen at -80°C , centrifuge cells at 15,000 rpm for 15 minutes at 4°C before starting extraction to ensure cell extracts are clear of any sediments or particulate matter. This may result in some loss of NAD/NADH .

11.1.2.1 Adherent cells: wash with cold PBS, trypsinize and harvest and transfer cells to a microcentrifuge tube.

11.1.2.2 Suspension cells: harvest and transfer cells to a microcentrifuge tube.

11.1.3 Centrifuge 5 minutes at 4°C at 2,000 rpm in a cold microcentrifuge to collect cells. Discard supernatant.

11.1.4 Wash cells twice with cold PBS by centrifugation at 4°C at 2,000 rpm for 5 minutes. Discard supernatant.

11.1.5 Spin down cells by microcentrifugation at 10,000 rpm for 1 minute. Remove as much supernatant as possible without disturbing pellet by aspiration.

11.1.6 NAD^+ Acid Extraction:

- 11.1.6.1 Vortex cell pellet gently.
- 11.1.6.2 Extract cells with 100 μ L NAD⁺ Extraction Solution by vortexing 3-4 times for 1 minute each with same time intervals or by homogenization using sonication (4 times for 5 seconds each on ice).
- 11.1.6.3 Let sample stand for 30 minutes on ice.
- 11.1.6.4 Add 100 μ L NAD⁺ Neutralization Solution to the acid extract and mix well by vortexing for neutralization.
- 11.1.6.5 Centrifuge the neutralized cell extract at 15,000 rpm for 5 minutes at 4°C.
- 11.1.6.6 Collect supernatant and transfer to a new tube. Label tube as **ACE** (Acid Extract) and keep on ice.

Δ Note: Make sure the final pH of the supernatant is 7.5-8.5. If is not within this range, adjust pH using NAD⁺ Neutralization Solution.

- 11.1.6.7 Optional: measure protein concentration to normalize data (pmol NAD⁺/mg protein).

11.1.7 NADH Alkaline Extraction:

- 11.1.7.1 Vortex cell pellet obtained from Step 11.1.5.
- 11.1.7.2 Extract cells with 100 μ L NADH Extraction Solution by vortexing 2-3 times for 1 minute each with same time intervals or by homogenization using sonication (4 times for 5 seconds each on ice).
- 11.1.7.3 Incubate sample at 60°C for 30 minutes to reduce viscosity.
- 11.1.7.4 Add 100 μ L NADH Neutralization Solution to the alkaline extract and mix well by vortexing for neutralization, then stand for at least 5 minutes on ice.
- 11.1.7.5 Centrifuge the neutralized cell extract at 15,000 rpm for 5 minutes at 4°C.
- 11.1.7.6 Collect supernatant and transfer to a new tube. Label tube as **ALE** (Alkaline Extract) and keep on ice.

Δ Note: Make sure the final pH of the supernatant is 7.5-8.5. If is not within this range, adjust pH using NADH Neutralization Solution.

- 11.1.7.7 Optional: measure protein concentration to normalize data (pmol NADH/mg protein).

Δ Note: We suggest using different volumes of sample to ensure readings are within the standard curve range.

12. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.
- Determination of NAD⁺ and NADH requires two separate samples (acid extract for NAD⁺ measurement and alkaline extract for NADH measurement), based on the character of NAD⁺: resistant to acidic condition and heat labile, and NADH: resistant to alkaline condition and relatively heat stable.

Δ Note: avoid mixing any reagents containing thiol groups (such as DTT or reduced glutathione) or alkyl amines in the sample as they will interfere with the assay.

12.1 NAD⁺/NADH Reaction mix:

12.1.1 Prepare 60 μL of Reaction mix. Prepare a master mix to ensure consistency.

Component	NAD ⁺ /NADH Reaction Mix (μL)
20X NAD/NADH Assay Buffer	5
WST-1	5
ADH	5
Diaphorase	5
Ethanol Solution	5
ddH ₂ O	35

Δ Note: keep reaction mix on ice and use within 30 minutes of preparation. Discard any unused mixture after use.

12.2 Reaction wells set up:

- Standard wells = 5 μL standard dilutions + 35 μL ddH₂O.
- ACE Sample wells (NAD⁺) = 1-5 μL samples (adjust volume to 40 μL /well with ddH₂O).
- ALE Sample wells (NADH) = 1-5 μL samples (adjust volume to 40 μL /well with ddH₂O).

12.3 NAD/NADH reaction:

- 12.3.1 Initiate reaction by adding 60 μL of Reaction Mix into each reaction well. Mix thoroughly by pipetting up and down.

The table below summarizes the reaction set up (Step 12.2 + 12.3):

Component	Standard well (μL)	ACE Sample well (μL)	ALE Sample well (μL)
NADH Standard	5	-	-
ACE sample test	-	1-5	-
ALE sample test	-	-	1-5
ddH ₂ O	35	35-39	35-39
NAD ⁺ /NADH Reaction Mix	60	60	60
Total reaction volume	100	100	100

12.4 Measurement:

- 12.4.1 Measure output at OD 450 nm on a microplate reader in kinetic mode for 30-90 minutes at 10 minute intervals at room temperature, protected from light.

Δ Note: Usually, appropriate reaction time is 60-90 minutes. Incubation reaction time will vary depending on the reaction conditions and preparation of cell extract. Decreasing the amount of cell extract in the assay may help to lengthen the time range.

Δ Note: Alternatively, after 60 minutes or the appropriate incubation time, reactions can be stopped by adding 50 μL of Stop Solution (1 N H₂SO₄, not provided) into each well and mix well. Then you can take a reading at OD 450 nm.

13. Calculations

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.
- Use only the linear rate for calculation.

13.1 Measure OD 450 nm signal:

- 13.1.1 Subtract OD 450 nm reading at time = 0 from all other reaction time readings.
- 13.1.2 Plot OD 450 versus reaction time.
- 13.1.3 Determine the reaction time range in which the increase in OD 450 nm is linear.
- 13.1.4 Fix an appropriate reaction time (usually, 60 minutes).
- 13.1.5 Take a reading with the absorbance at OD 450 nm.

13.2 Calculate NAD⁺ and NADH in the sample:

- 13.2.1 Average the duplicate reading for each standard and sample.
- 13.2.2 Subtract the mean absorbance value of the blank (Standard #8) from all standard and sample readings. This is the corrected absorbance.
- 13.2.3 Plot the corrected absorbance values for each standard as a function of the final concentration of NADH.
- 13.2.4 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).
- 13.2.5 Apply the corrected sample OD reading to the standard curve to get NAD⁺ or NADH amount in the sample wells.

13.2.6 Concentration of NAD⁺ or NADH (pmol/cell number) in the test samples is calculated as:

$$\text{NAD concentration} = \left(\frac{\text{ACE} * 2 * 10^5}{\text{cell number}} \right) * D$$

$$\text{NADH concentration} = \left(\frac{\text{ALE} * 2 * 10^5}{\text{cell number}} \right) * D$$

Where:

ACE = NAD⁺ concentration in the ACE test sample calculated from standard curve (nM).

ALE = NADH concentration in the ALE test sample calculated from standard curve (nM).

D = sample dilution factor if sample is diluted to fit within the standard curve range.

NAD⁺ and NADH concentration can be expressed in pmol/mg protein if a protein quantification assay has been previously performed.

13.2.7 NAD/NADH ratio is calculated as:

$$\text{NAD/NADH Ratio} = \frac{\text{NAD concentration (pmol/10}^6 \text{ cells)}}{\text{NADH concentration (pmol/10}^6 \text{ cells)}}$$

14. Typical Data

Data provided for demonstration purposes only.

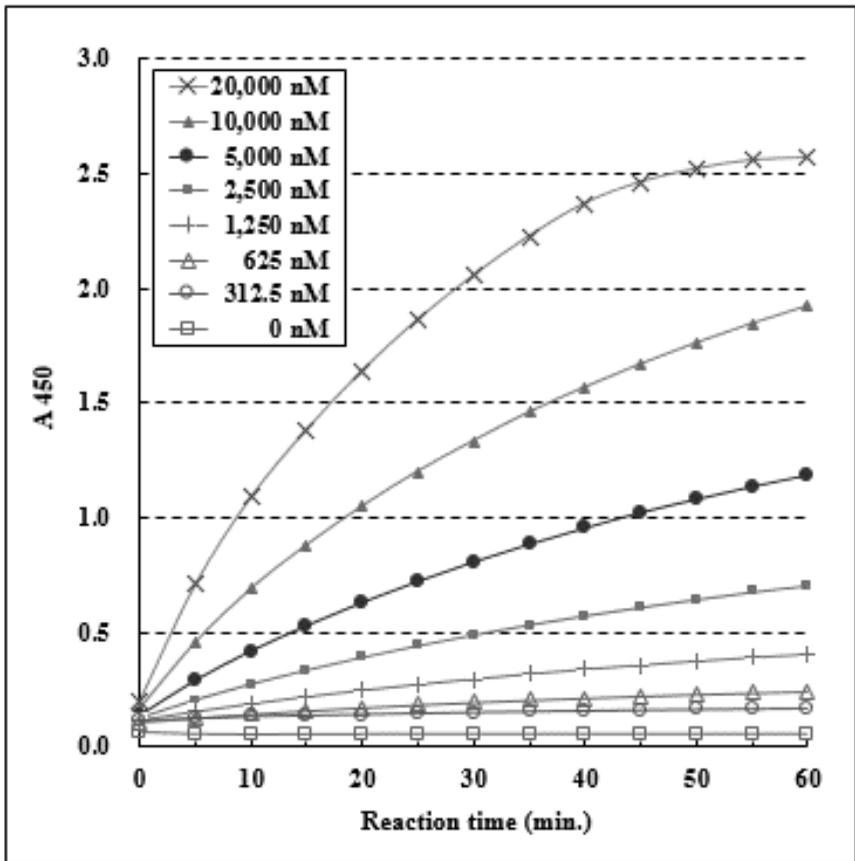


Figure 1. Time time course curve curve of NADH standards.

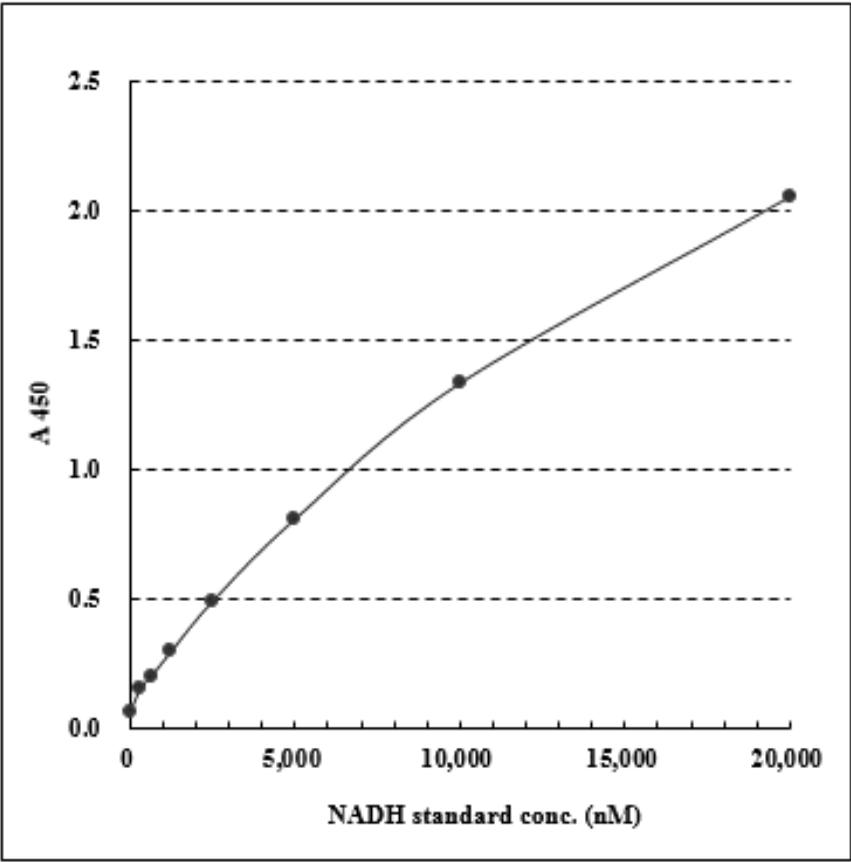


Figure 2. Typical standard curve of NADH concentration.

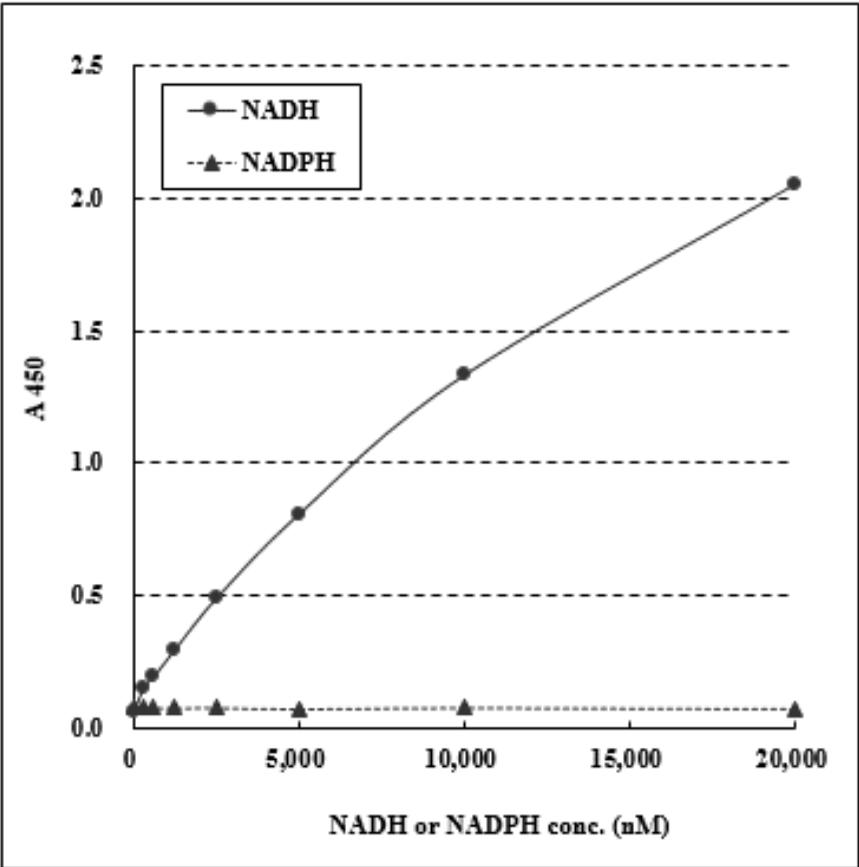


Figure 3. Specific detection and measurement of NADH, not NADPH (As low as 312.5 nM of NADH can be detected with 30 minutes incubation time (n=2), there is no response to NADPH.)

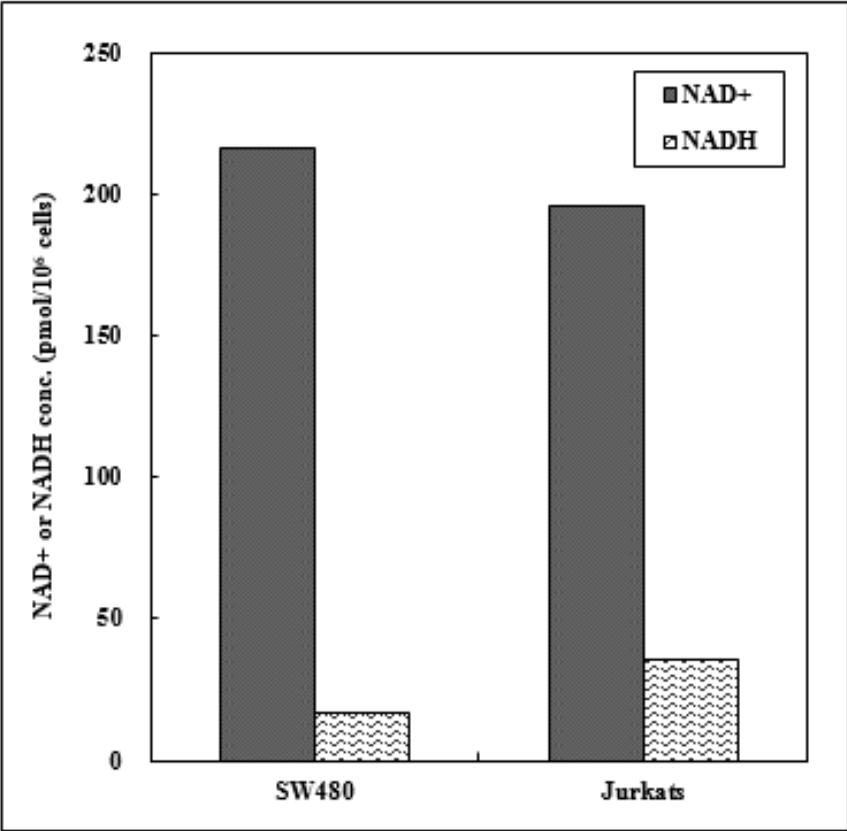


Figure 4. NAD⁺ and NADH concentrations in cell extracts of SW480 and Jurkat cells.

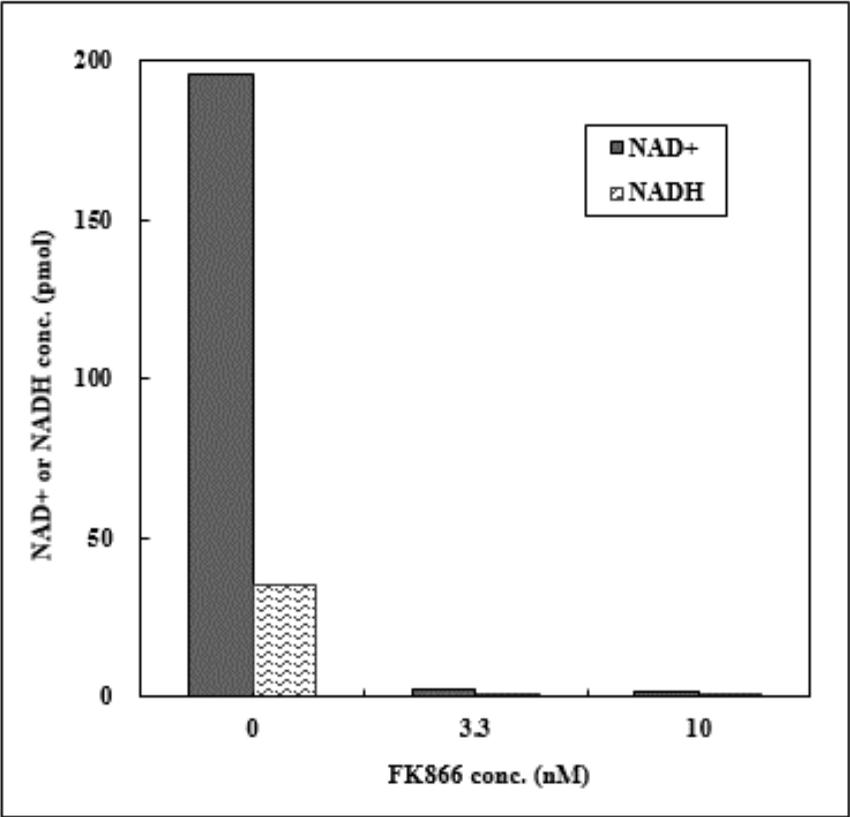


Figure 5. NAD⁺ and NADH concentrations in cell extracts of Jurkat cells treated with NAMPT specific inhibitor FK866 at indicated concentrations.

15. Troubleshooting

Problem	Reason	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at assay temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different microplate	Colorimetric: clear plates Fluorometric: black wells/clear bottom plates Luminometric: white wells/clear bottom plates
Sample with erratic readings	Enzyme cycling reaction inhibited by low pH	Use NAD ⁺ or NADH neutralization solution to adjust sample to pH 7.5-8.5
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

Problem	Reason	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 μ L) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions described in the protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

16. Interferences

These chemical or biological materials will cause interferences in this assay causing compromised results or complete failure:

- Reagents containing thiol groups such as DTT or reduced glutathione
- Reagents containing alkyl amine

17. FAQs

Q. Can I use sodium carbonate-based buffers for the NAD⁺ or NADH extraction?

A. We do NOT recommend to use sodium carbonate-based buffers as NAD⁺/NADH extraction solution because these extracts may give inappropriate NAD⁺ and NADH concentrations and ratio.

Q. What is the difference between this kit and NAD/NADH Assay Kit (Colorimetric) (ab65348)?

A. Because this kit relies on NAD⁺ and NADH extraction, is more sensitive than ab65348 is therefore recommended for cell lines where low amount of NAD⁺ or NADH is expected.

18. Notes

Technical Support

Copyright © 2017 Abcam, All Rights Reserved. The Abcam logo is a registered trademark. All information / detail is correct at time of going to print.

Austria

wissenschaftlicherdienst@abcam.com | 019-288-259

France

supportscientifique@abcam.com | 01.46.94.62.96

Germany

wissenschaftlicherdienst@abcam.com | 030-896-779-154

Spain

soportecientifico@abcam.com | 91-114-65-60

Switzerland

technical@abcam.com

Deutsch: 043-501-64-24 | Français: 061-500-05-30

UK, EU and ROW

technical@abcam.com | +44(0)1223-696000

Canada

ca.technical@abcam.com | 877-749-8807

US and Latin America

us.technical@abcam.com | 888-772-2226

Asia Pacific

hk.technical@abcam.com | (852) 2603-6823

China

cn.technical@abcam.com | 400 921 0189 / +86 21 2070 0500

Japan

technical@abcam.co.jp | +81-(0)3-6231-0940

Singapore

sg.technical@abcam.com | 800 188-5244

Australia

au.technical@abcam.com | +61-(0)3-8652-1450

New Zealand

nz.technical@abcam.com | +64-(0)9-909-7829