

## Product Information

### NAD/NADH Quantitation Colorimetric Kit

#### I. Kit Contents:

Components	K2036-100 100 assays	Cap Color	Part Number
NADH/NAD Extraction Buffer	50 ml	NM	K2036-C-1
NAD Cycling Buffer	15 ml	NM	K2036-C-2
NAD Cycling Enzyme Mix	1 vial	Green	K2036-C-3
NADH Developer	1 vial	Purple	K2036-C-4
Stop Solution	1.2 ml	Red	K2036-C-5
NADH Standard	1 vial	Yellow	K2036-C-6

#### II. Introduction:

Nicotinamide adenine dinucleotide (NAD) is a coenzyme present in all living cells and exists in two forms: NAD<sup>+</sup> and NADH. NAD plays important roles in energy transforming and redox state of cells or tissues.

The NAD/NADH Quantitation Colorimetric Kit provides a sensitive and convenient way for detection of the intracellular nucleotides: NADH, NAD and their ratio. The NAD Cycling Enzyme Mix specifically recognizes NADH/NAD in an enzyme cycling reaction, which are not required to purify from samples. The enzyme cycling reaction significantly increases the detection specificity and sensitivity. By comparing with standard NADH, NADH or NADt (NAD and NADH) can be easily quantified. The assay specifically detects NAD and NADH, but not NADPH or NADP.

#### III. Application:

Measurement of NADH in various tissues/cells.

Analysis of metabolism in various cells.

#### IV. Sample Type:

Animal tissues: liver, kidney etc.

Cell culture: adherent or suspension cells.

Serum.

Urine.

#### V. User Supplied Reagents and Equipment:

96-well clear plate with flat bottom.

Multi-well spectrophotometer.

#### VI. Storage and Handling:

Store kit at -20°C, protected from light. Briefly centrifuge small vials prior to opening. Read entire protocol before performing the assay.

## VII. Reagent Reconstitution and General Consideration:

NADH/NAD Extraction Buffer: Warm NADH/NAD Extraction Buffer to room temperature before use. Store at 4°C or -20°C.

NAD Cycling Buffer: Warm NAD Cycling Buffer to room temperature before use. Store at 4°C or -20°C.

NAD Cycling Enzyme Mix: Reconstitute with 220 µl NAD Cycling Buffer. Aliquot enough NAD Cycling Enzyme mix (2 µl per assay) for the number of assays to be performed in each experiment and freeze the stock solution immediately at -70°C for future use. The enzymes are stable for up to 2 months at -70°C after reconstitution.

NADH Developer: Reconstitute NADH developer with 1.2 ml of ddH<sub>2</sub>O. Pipette up and down several times to completely dissolve the pellet into solution (don't vortex). Store at -20°C, protected from light. Use within 2 months.

NADH Standard: Reconstitute with 200 µl pure DMSO to generate 1 nmol/µl NADH Standard solution. Store at -20°C. Use within 2 months.

Note: Ensure that the NAD Cycling Buffer is at room temperature before use. Keep other enzymes on ice during the assay and protect from light.

## VIII. NAD/NADH Assay Protocol:

1. Sample Preparation: Wash cells with cold PBS. Pellet  $2 \times 10^5$  cells for each assay in a micro-centrifuge tube (2000 rpm for 5 min.) & extract with 400 µl of NADH/NAD Extraction Buffer by freeze/thaw two cycles (20 min. on dry-ice, then 10 min. at room temperature), or by homogenization. Vortex the extraction for 10 sec. Centrifuge at 14000 rpm for 5 min. Transfer the extracted NADH/NAD supernatant into a labeled tube. For tissues, weigh ~20 mg tissue & wash with cold PBS. Homogenize in 400 µl of NADH/NAD Extraction Buffer in a micro-centrifuge tube. Centrifuge at 14000 rpm for 5 min. Transfer the extracted NADH/NAD supernatant into a new tube. To detect total NADt (NADH and NAD), transfer 50 µl of extracted samples into labeled 96-well plate. To detect NADH, NAD needs to be decomposed before the reaction. To decompose NAD, aliquot 200 µl of extracted samples into eppendorf tubes. Heat to 60°C for 30 min. in a water bath or a heating block. Under this condition, all NAD will decompose, while NADH will still be intact. Cool samples on ice. Quick spin the samples to remove precipitates if precipitation occurs. Transfer 50 µl of NAD decomposed samples into labeled 96-well plate.

Notes:

- Cell or tissue lysates may contain enzymes that consume NADH rapidly. We suggest removing these enzymes by filtering the samples through 10 kDa molecular weight cut off filters before performing the assay.
- For unknown samples, we suggest performing a pilot experiment & testing different sample dilutions with the extraction buffer to ensure the readings are within the Standard Curve range.
- For samples having high background, prepare parallel well(s) containing same amount of sample as in the test well. Adjust the volume to 50 µl/well with NADH/NAD Extraction Buffer.
- Endogenous compounds may interfere with the reaction. To ensure accurate determination of NADH in the test samples, we recommend spiking samples with a known amount of Standard (60 pmol).

2. Standard Curve Preparation: Dilute 10 µl of 1 nmol/µl NADH standard with 990 µl NADH/NAD Extraction Buffer to generate 10 pmol/µl standard NADH. Add 0, 2, 4, 6, 8, 10 µl of the diluted NADH standard into labeled 96-well plate in duplicate to generate 0, 20, 40, 60, 80, 100 pmol/well standard. Make the final volume to 50 µl with NADH/NAD extraction buffer. Note: diluted NADH solution is unstable, must be used within 4 hours.

3. Reaction Mix: Prepare a NAD Cycling Mix for each reaction:

	Reaction Mix	Background Control Mix
NAD Cycling Buffer	98 µl	100 µl
NAD Cycling Enzyme Mix	2 µl	---

Mix well and add 100 µl of the mix into each well of NADH Standard and samples. Mix. Incubate the plate at room temperature for 5 min. to convert NAD to NADH.

For samples having high background, add 100 µl of Background Control Mix to sample background control well(s).

4. Measurement: Add 10 µl NADH developer into each well. Let the reaction cycling at room temperature for 1 to 4 hrs or longer depending on the reading (OD<sub>450 nm</sub>). Read the plate at OD<sub>450 nm</sub>. The plate can be read multiple times while the color is being developed. The reactions can be stopped by adding 10 µl of Stop Solution into each well and mix well. The color should be stable for 48 hrs in a sealed plate after addition of Stop Solution.

5. Calculation: for unspiked samples, apply the sample readings to NADH standard curve. The amount of NADt or NADH in the sample wells can then be calculated. Divide the NADt or NADH amount by the sample amount (e.g. cell number or extract protein amount) you added into the sample wells. The concentration of NADt or NADH can be expressed in pmol/10<sup>6</sup> cells or ng/mg protein (NADH molecular weight 663.4).

Note: For spiked samples, correct for any sample interference by subtracting the sample reading from spiked sample reading.

For spiked samples, NADt or NADH amount in sample well = (OD<sub>sample(corrected)</sub>) / [(OD<sub>sample+ NADH Std(corrected)</sub>) - (OD<sub>sample(corrected)</sub>)] \* NADH Spike (pmol)

NAD/NADH Ratio is calculated as: (NADt - NADH) / NADH

## Frequently Asked Questions

1. In the protocol, it is mentioned that to detect NADH, the NAD needs to be decomposed before the reaction. Does it mean that 1) there is no NAD present in the well, and 2) if the NAD cycling mix is added at this step, no reaction should be observed? Could you also summarize the way the kit works? How and when is the NAD measured? How and when is the NADt quantified?

This kit runs on a simple principle. You can either measure the total NAD+NADH or just the NADH. To measure the NAD, you need to subtract NADH levels from the NADt levels. This kit will only measure NADH. So if NADt has to be measured, all the existing NAD has to be converted to NADH prior to detection. This is what the cycling enzyme does. If the levels of NADH only are to be measured, the NAD needs to be decomposed, which is done at an elevated temp.

2. I bought the kit and have another question concerning the protocol. It says, for the detection of NADH I need to do the decomposing step on 60 C first. The next step would be the NAD Cycling, where NAD is transformed into NADH. The protocol does not mention anything about leaving this step out if I only want to detect NADH. I guess it is useless if I decomposed NAD in the step before? So after decomposing NAD I'd directly jump to adding the developer, is that correct? Another question, the protocol says incubation for 1 to 4 hours: will reaction results after 1 hour differ much from results after 4 hours? I don't understand how long I have to incubate the samples with the developer to have my end-results.

It seems logical to ignore the step of NAD conversion to NADH if you have already decomposed the NAD, but I would still recommend you to follow the protocol exactly without deleting any steps. The NAD conversion to NADH adds some volume to each of the samples, as well as the standards. You definitely want this volume to be consistent between the standards and samples for comparing between them. Therefore, please do all the steps even if you want to assay just for NADH.

Your signals from 1 to 4 hrs of final incubation will ideally increase. Within that time range, whenever you are comfortable and satisfied with the signals, you can add the stop solution to terminate any more colour development.

3. I would like to know something more specific about the reactions, namely what exactly is happening in the cycling reaction. From what I understand, at that time we will have two different tubes for the same sample, one for NADt and one for NADH. What is the function of the NAD cycling mix?

In the cycling reaction, all the NAD is getting converted to NADH. The NAD cycling mix will help in the conversion of NAD to NADH. Note that you can detect only NADH in this assay, since there is no NAD developer included. Therefore to measure total NADH, you will need to convert the existing NAD to NADH, and then when you develop the reaction, you will get the total NADH. To measure only NAD, you will decompose the NAD, measure for NADH and then subtract that from the total NADH reading.

4. Stop solution, should it be added before or after the final measurement? Usually the stop solution is added before, so all the reactions stop at the same, and the measurements are not biased. However, the way it is written in the protocol, it seems the readings are done before the stop solution is added.

Yes, the stop solution is added before the final measurement. You just keep developing the color until it falls within the linear range of the standard curve (which you can do only when you measure the absorbance). Once this colour is reached, you add the stop solution to all wells and then take the final measurement. Thus there is no measurement bias introduced.

5. If we choose to lyse cells by homogenizing instead of freeze-thaw, what kind of device or method is the most suitable?

Please use a Dounce homogenizer. About 30 - 50 passages should be good for the homogenization. You can perform a microscopic examination to ascertain the homogenization. If required, please do 10 - 20 more passages.

6. Can this kit be used with samples like bacteria, plants, drosophila, yeast etc?

We have optimized the kit with mammalian samples. However, theoretically these kits should work with samples from multiple species/sources. Since the optimal conditions depend on the sample type, the protocol has to be adapted to fit the samples for efficient results. Please refer to this kit's citations to see what kind of samples have been used with this kit other than mammalian samples.

7. Can we use frozen samples with this assay?

Fresh samples are always preferred over frozen samples. However, frozen samples can also be used, provided, they were frozen right after isolation, were not freeze thawed multiple times (for which we recommend aliquoting the samples before freezing) and have been frozen for relatively short periods.

8. Can we use a different wavelength than recommended for the final analysis?

It is always recommended to use the exact recommended wavelength for the most efficient results. However, most plate readers have flexibility in their band width of detection in increments of  $\pm 10$  nm. Depending on this flexibility range, you can deviate from the recommended wavelengths within limits.

9. What is the exact volume of sample required for this assay?

There is no specific volume we can recommend for the amount any sample to be used since it is completely sample concentration and quality based. You have to do a pilot experiment with multiple sample volumes to determine the optimal volume which gives a reading within the linear range of the standard curve. Please refer to the citations for this product to see what other clients have used with similar sample types.

11. What is the shelf life of this kit?

This kit is good for 12 months from the date of shipment in the unopened form when stored at the appropriate temperature and appropriate conditions. After opening and reconstitution, some of the components in this kit are good for 2 months at  $-20^{\circ}\text{C}$ . Please refer to the datasheet for storage information and shelf life of each of the components.

12. Why are my standard curve values lower than those shown on the datasheet?

There are multiple factors which influence the signals like the incubation times, room temperature, handling etc. In general, to increase the value of the standards, you can increase the incubation time. As long as the standard curve is linear, it should be fine to use, since all of your samples will also be measured under the same conditions on this curve.

13. How do I normalize my samples against protein concentration

You can use a protein quantitation assay on the supernatants you get from cell/tissue lysates or with any other liquid sample in the assay buffer.

14. Can we use an alternate buffer for sample preparation (cell lysis, sample dilutions etc)?

Our assay buffers are optimized for the reactions they are designed for. They not only contain some detergents for efficient lysis of your cells/tissue, but also contain some proprietary components required for the further reactions. Therefore, we highly recommend using the buffers provided in the kit for the best results.

15. Should I make a standard curve for every expt I do, or is one curve/kit enough?

Yes, I would strongly recommend you to do the standards every time you do the expt. There is always a chance that something was done differently that day and we do not want any conditions to differ between standards and samples.

**For research use only! Not to be used in humans.**

## **Our promise**

If the product does not perform as described on this datasheet, we will offer a refund or replacement. For more details, please visit <http://www.apexbt.com/> or contact our technical team.

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