

Certificate of Analysis

Analysis type: NAD+ purity test

Customer: Inside Out Biotech Ltd.

Sample number: 2

Sample state: dry powder in clear sealed glass bottles

Sample reception date: 11.06.2024

Storage conditions before analysis: -20°C, light protected

Purpose of the analysis is to determine content of physiologically active NAD+ as anhydrous base in each bottle.

Contents of NAD+ purity test:

1. Visual inspection of the sample
2. UV-Vis Spectroscopy analysis of the sample to determine concentration of NAD+-related molecules
3. Functional assay to determine content of physiologically active NAD+ in mg in the sample
4. Summary

1. Visual inspection the sample

Description

Each bottle contained dry fine crystalline powder of white color. Content of each bottle was weighted and reconstituted in 5 ml of deionized water followed by measurement of resulting volume.

Sample	Color of the cap	Weight of the powder	Volume of obtained solution	Comment on the appearance of the solution
1	white	0,98 g	5,41 ml	Solution had slightly yellow hue with undissolved particles
2	red	0,61 g	5,2 ml	Colorless clear solution



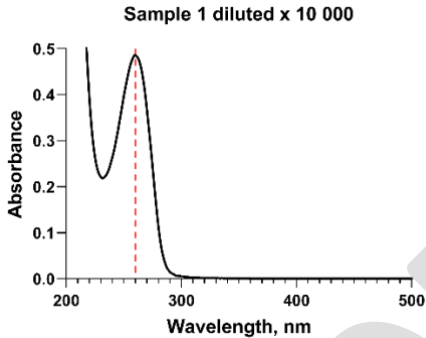
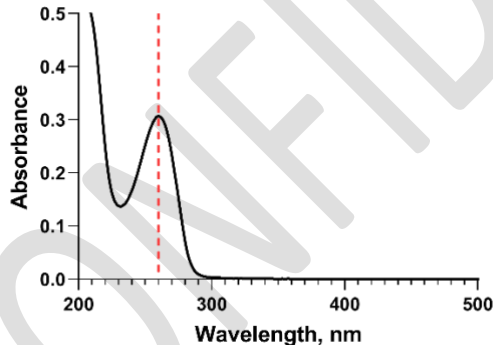
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2. UV-Vis Spectroscopy analysis of the sample to determine concentration of NAD⁺ related molecules

Description

Provided solutions were diluted 10 000 times with deionized water and UV-Vis spectra was measured using Shimadzu UV-2401pc Spectrophotometer and compared with Reference spectra of NAD⁺ Sigma Cat#N1636. UV-VIS spectra of NAD⁺-related compounds in aqueous solutions have absorbance peak at 260nm with extinction coefficient $\lambda=18 \text{ mM} \cdot \text{cm}^{-1}$, which is used to determine concentration of pool of NAD⁺-related molecules.

Result: All samples showed NAD⁺-related characteristic absorbance peak with maximum at 260 nm (shown on the left panel in the Table below).

Sample ID	Optical spectrum of NAD ⁺ -related content	Parameters
1	 <p>Sample 1 diluted x 10 000</p>	OD at 260 nm = 0,486 Concentration - 270 mM
2	 <p>Sample 2 diluted x 10 000</p>	OD at 260 nm = 0,307 Concentration = 170 mM

3. Functional assay to determine content of physiologically active NAD⁺ fraction in the sample

Description

To examine whether determined concentration of NAD⁺ represent physiologically active form of the compound we used our proprietary calibrated NADMED assay for NAD⁺. In this assay NAD⁺-specific enzyme uses selectively NAD⁺ to produce colored substance, which light absorbance is linearly proportional to NAD⁺ concentration in the added solution. If solution contains, for example, ADP-ribose, which is a moiety in NAD⁺ molecule with UV-VIS absorption spectra similar to NAD⁺, the enzyme will not react with it and, therefore, we will see lower signal than expected from the spectroscopy measurement of the concentration.



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To perform this assay we prepared 100 000 dilution of each of provided samples using proprietary buffer stabilizing specifically NAD⁺ and run the assay according to the protocol we use for measurement of NAD⁺ concentration in biological samples.

Sample ID	Response in the NAD ⁺ assay	Parameters
1		Concentration in x100 000 diluted solution - 2,42 μ M Concentration in original solution- 242 mM
2		Concentration in x100 000 diluted solution - 1,60 μ M Concentration in original solution- 160 mM

4. Summary

Based on measured: 1) weight of the powder in each bottle; 2) concentration of physiologically active NAD⁺ and 3) molecular weight of anhydrous NAD⁺ (663,43 g/mol) purity grade was calculated.

Sample ID	Weight of the powder, g	Amount of physiologically active NAD ⁺ , g	Purity of the physiologically active NAD ⁺ in the powder, %
1	0,98	0,868	88,6
2	0,61	0,554	90,9

Report issue date: 19.06.2024

Report validated by: Liliya Euro, PhD, Chief Scientist in NADMED Oy

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