Version 3 Last updated 30 October 2023

ab235945 5'-Nucleotidase (CD73) Activity Assay Kit (Colorimetric)

For the measurement of 5'-nucleotidase activity in tissue and cell lysates.

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

PLEASE NOTE: With the acquisition of BioVision by Abcam, we have made some changes to component names and packaging to better align with our global standards as we work towards environmental-friendly and efficient growth. You are receiving the same high-quality products as always, with no changes to specifications or protocols.

Table of Contents

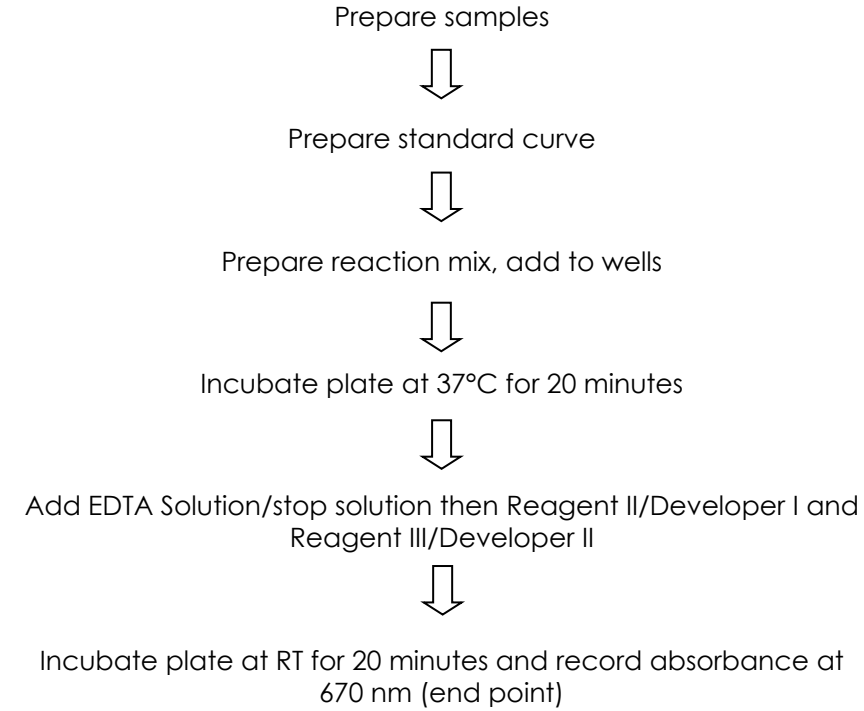
1. Overview

5'-Nucleotidase (CD73) Activity Assay Kit (Colorimetric) (ab235945) is a simple two-step end-point assay for the measurement of 5'-Nucleotidase activity in various tissues/cells. This assay relies on the Berthelot's test for quantification of ammonia.

In this assay, the action of 5'-nucleotidase on the substrate generates a product, which releases ammonia in presence of the converter. Reagent II/Developer I and Reagent III/Developer II are then used to quantify the released ammonia through increase in absorbance at 670 nm.

This assay can detect as low as 0.2 mU of 5'-NT. Since non-specific enzymes like alkaline phosphatase can give a positive signal in this assay, 5'-NT inhibitor may be used to completely inhibit 5'-nucleotidase and distinguish from the signal from non-specific enzymes.

The assay kit also includes 5'- Nucleotidase (5'-NT) enzyme for use as positive control.



2. Materials Supplied and Storage

Store kit at -20°C in the dark immediately on receipt and check below for storage for individual components.

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

Avoid repeated freeze-thaws of reagents.

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
5'-NT Buffer/5'-NT Assay Buffer	25 mL	-20°C	-20°C
5'-NT Convertor	1 vial	-20°C	-80°C
Reagent II/5'-NT Developer I	8 mL	-20°C	-20°C
Reagent III/5'-NT Developer II	4 mL	-20°C	-20°C
5'-NT Inhibitor	250 µL	-20°C	-20°C
5'-NT Positive Control	1 vial	-20°C	-80°C
EDTA Solution/5'-NT Stop Solution	500 µL	-20°C	-20°C
5'-NT Substrate	1 vial	-20°C	-20°C
Ammonium Standard II/NH_4^+ Standard (100 mM)	100 µL	-20°C	-20°C

3. 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 O.D. 670 nm
- 96 well plate with clear flat bottom
- Incubator / water bath that can be heated to 37°C
- Dounce homogenizer (if using tissue)

4. General guidelines, precautions, and troubleshooting

Please observe safe laboratory practice and consult the safety datasheet.

For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide:
www.abcam.com/assaykitguidelines

For typical data produced using the assay, please see the assay kit datasheet on our website.

5. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

5.1 5'-NT Buffer/5'-NT Assay Buffer

1. Ready to use as supplied.
2. Warm to room temperature before use.

5.2 5'-NT Convertor

1. Stable for at least 3 months when stored at -20 °C.
2. Reconstitute with 220 µl 5'-NT Buffer/5'-NT Assay Buffer before use.
3. Gently pipette up and down to dissolve completely and then centrifuge briefly.
4. Aliquot and store at -80°C. Use within two months.
5. Keep on ice while in use.

5.3 Reagent II/5'-NT Developer I

1. Ready to use as supplied.

5.4 Reagent III/5'-NT Developer II

1. Ready to use as supplied.

5.5 5'-NT Inhibitor

1. Ready to use as supplied.

5.6 5'-NT Positive Control

1. Lyophilized enzyme is stable for at least 6 months when stored at -20 °C.
2. Add 22 µl 5'-NT Buffer to the Positive Control and mix thoroughly.
3. Aliquot and store at –80°C. Use within two months.
4. Keep on ice while in use.

5.7 EDTA Solution/5'-NT Stop Solution

1. Ready to use as supplied.

5.8 5'-NT Substrate

1. Reconstitute with 1.1 ml 5'-NT Buffer/5'-NT Assay Buffer.
2. Aliquot and store at -20°C.
3. Reconstituted substrate is stable for at least 2 months.

5.9 Ammonium Standard II/NH_4^+ Standard (100 mM)

1. Ready to use as supplied.

6. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

1. Prepare 1 mM Ammonium Standard solution by diluting the provided 100 mM standard (add 10 µL 100 mM standard to 990 µL dH_2O).
2. Using 1 mM Ammonium Standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	1 mM Standard (µL)	5'-NT Buffer/Assay Buffer (µL)	Final volume standard in well (µL)	End amount of 1mM Ammonium standard in well (nmol/well)
1	0	200	100	0
2	4	196	100	2
3	8	192	100	4
4	12	188	100	6
5	16	184	100	8
6	20	180	100	10
7	30	170	100	15

Each dilution has enough standard to set up duplicate readings (2 x 100 µL).

7. 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 for the most reproducible assay. 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. Be aware, however, that this might affect the stability of your samples and the readings can be lower than expected. Avoid multiple freeze-thaws.

7.1 Tissue/Cells:

1. Rapidly homogenize tissue (10 mg) or cells (1 x 10^6) with 100 µL ice cold 5'-NT Buffer/5'-NT Assay Buffer, and keep on ice for 10 minutes.
2. Centrifuge at 10,000 x *g* for 10 minutes at 4°C and transfer the supernatant to a fresh tube.
3. Determine protein concentration using preferred method. Protein concentration should range between 1-20 mg/mL. Concentrated samples may be diluted with 5'-NT Buffer/5'-NT assay buffer.
4. Aliquot and store lysates at -80°C unless being used immediately.
5. Use 5-20 µL sample per well.
6. Prepare three identical wells for each sample labelled "Sample Background Control" (BC), "Sample" (S) and "Sample + Inhibitor" (SI).
7. For SI well, add 5 µL 5'-NT Inhibitor in addition to sample.
8. Adjust volume in each well to 50 µl with 5'-NT Buffer/5'-NT Assay Buffer.
9. For positive control (PC) and inhibitor control (IC), add to two wells 2 µL of 5'-NT Positive Control into desired well(s), add to the inhibitor control well 5 µl inhibitor and adjust the final volume of the wells to 50 µL with 5'-NT Buffer/5'-NT Assay Buffer.

8. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature just prior to use and gently agitate.
- Assay all standards, controls and samples in duplicate.

Δ Note: If you suspect your samples contain substance that can generate background, set up Sample Background Controls to correct for background noise.

8.1 Reaction mix:

1. For each well prepare 50 µL of Reaction Mix and Background Mix for each reaction. Prepare a master mix to ensure consistency.

Component	Reaction Mix (µL)	Background Reaction Mix (µL)
5'-NT Buffer/5'-NT Assay Buffer	38	48
5'-NT Converter	2	2
5'-NT Substrate	10	---

2. Add 50 µL of Reaction Mix into each sample and positive control wells.
3. Add 50 µL of Background Reaction Mix into the background control sample wells.
4. Total volume in wells should now be 100 µL.
5. Incubate the plate at 37°C for 20 minutes.

Δ Note: If low enzyme activity is observed or expected in samples, incubation time may be increased.

8.2 Measurement:

1. Add 4 µL of EDTA Solution/stop solution to each well followed by 80 µL of Reagent II/5'-NT Developer I and 40 µl of Reagent III/5'-NT Developer II.

2. Incubate at RT for 15-20 minutes and record absorbance at 670 nm (end point).

Δ Note: Do not premix the reagents. They should be added to the well separately.

Δ Note: Do not let the plate sit for more than 20 minutes.

Δ Note: Turbidity upon addition of Developer solution is normal and will disappear in few minutes. The total volume in every well (i.e. samples, background controls and standards) should be 224 µL.

9. Data Analysis

1. Average the duplicate reading for each standard, control and sample.
2. Subtract the mean value of the blank (Standard #1) from all standards, controls and sample readings. This is the corrected absorbance.
3. If significant, subtract the sample background control from sample readings.
4. Plot the corrected values for each standard as a function of the final concentration of NH_4^+.
5. 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).
6. Apply the corrected sample O.D. reading to the standard curve to get detected activity (B) amount in the sample wells.
7. Concentration of NH_4^+ in mU/mg in the test samples is calculated as:

$$\text{Detected activity} = \frac{B}{(\Delta t \text{ X } p)}$$

Where:

B = amount of NH_4^+ in the sample well calculated from standard curve in nmol.

Δt = reaction time i.e. 20 minutes

p = sample protein content added (mg)

Specific 5'-NT activity in sample = detected activity in S – detected activity in SI

Unit Definition: One unit of 5'-NT is the amount of enzyme that generates 1.0 µmol of NH_4^+ per minute at pH 7.4 at 37°C.

10. Typical Data

Data provided **for demonstration purposes only.**

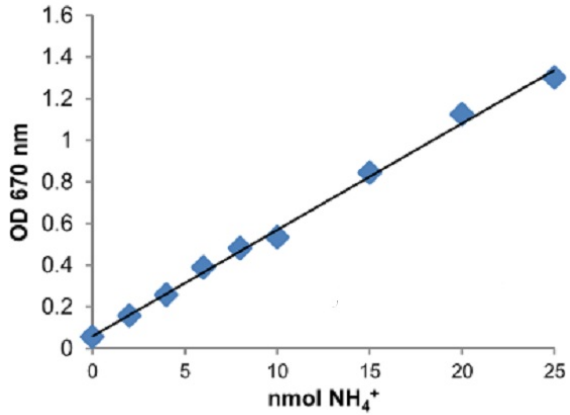


Figure 1. NH_4^+ standard curve.

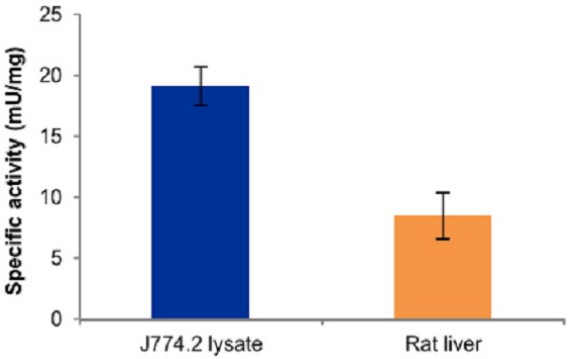


Figure 2. 5'-NT specific activity in J774.2 mouse macrophage (60 µg protein) cell line lysate and rat liver tissue lysate (40 µg protein).

11. Notes

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