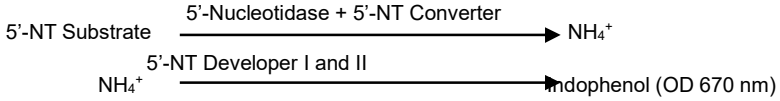
5’-Nucleotidase (CD73) Activity Assay Kit (Colorimetric) (#BN01150)

(Catalog # BN01150; 100 assays; Store at -20°C)

I. Introduction:

5’-Nucleotidase (5’-NT), also known at CD73 (EC 3.1.3.5) is an enzyme located in the plasma membrane. It converts extracellular nucleotides like 5’-AMP to their corresponding nucleosides, through phosphorylitic cleavage. This conversion facilitates uptake of the nucleosides through nucleoside receptors into the cell, where they can again be phosphorylated to generate nucleotides and contribute to the nucleotide pool, inside the cell. 5’-NT levels are elevated in hepatic diseases such as viral hepatitis, alcoholic liver disease and cirrhosis. Assay Genie’s 5’-Nucleotidase Activity Kit is a simple two-step end point assay that 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. Developer I and 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.



II. Applications:

- Measurement of 5’-Nucleotidase activity in various tissues/cells

III. Sample Type:

- Animal tissues lysate: eg. liver
- Cell lysate
- Recombinant enzyme, purified protein

IV. Kit Contents:

Components	BN01150	Cap Code	Part Number
5’-NT Assay Buffer	25 ml	WM	BN01150-1
5’-NT Substrate	1 vial	Blue	BN01150-2
5’-NT Convertor	1 vial	Green	BN01150-3
5’-NT Inhibitor	250 µl	Orange	BN01150-4
5’-NT Stop Solution	500 µl	Red	BN01150-5
5’-NT Developer I	8 ml	Amber	BN01150-6
5’-NT Developer II	4 ml	Clear	BN01150-7
NH_4^+ Standard (100 mM)	100 µl	Yellow	BN01150-8
5’-NT Positive Control	1 vial	Purple	BN01150-9

V. User Supplied Reagents and Equipment:

- 96-well clear plate with flat bottom
- Multi-well spectrophotometer
- Incubator / water bath that can be heated to 37ºC

VI. Storage Conditions and Reagent Preparation:

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

- **5’-NT Buffer:** Warm to room temperature before use.
- **5’-NT Substrate:** Reconstitute with 1.1 ml 5’-NT Assay Buffer. Aliquot and store at -20°C. Reconstituted substrate is stable for at least 2 months.
- **5’-NT Convertor:** Stable for at least 3 months when stored at -20 ºC. Reconstitute with 220 µl 5’-NT Assay Buffer before use. Gently pipette up and down to dissolve completely and then centrifuge briefly. Aliquot and store at -80°C. Use within two months. Keep on ice while in use.
- **5’-NT Positive Control:** Lyophilized enzyme is stable for at least 6 months when stored at -20 ºC. Add 22 µl 5’-NT Buffer to the Positive Control and mix thoroughly. Aliquot and store at –80°C. Use within two months. Keep on ice while in use.
- All other components are ready to use after thawing.

VII. 5’-Nucleotidase Activity Assay Protocol:

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

to two wells 2 µl of 5'-NT Enzyme 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 Assay Buffer.

Note: For unknown samples, we suggest testing several doses to ensure the readings are within the Standard Curve range.

2. **NH_4^+ Standard Curve Generation:** Prepare 1 mM Ammonium Standard solution by diluting the provided 100 mM standard (add 10 µl 100 mM standard to 990 µM dH_2O). Mix well. Add 0, 2, 4, 6, 8, 10 and 15 µl into a series of wells in a clear 96 well plate. Add 5'-NT Assay Buffer to each well and bring up the total volume to 100 µl to generate 2, 4, 6, 8, 10 and 15 nmol / well of ammonium standard.
3. **Reaction Mix:** Mix enough reagents for the number of assays to be performed. Add BC Mix to "Sample background control" wells and Reaction Mix to all other wells. For each well, prepare 50 µl:

	BC Mix	Reaction Mix
5'- NT Assay Buffer	48 µl	38 µl
5'- NT Converter	2 µl	2 µl
5'- NT Substrate	-	10 µl

Add the reaction mix to wells of the 96-well plate containing the samples and positive control. wells. *The volume at this stage in every well (i.e. S, BC, SI, PC, IC and standards) is 100 µl.*

4. **Incubation:** Incubate the plate at 37°C for 20 minutes.

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

5. **Measurement: Measurement:** Add 4 µl of stop solution to each well followed by 80 µl of 5'-NT Developer I and 40 µl of 5'-NT Developer II. Incubate at RT for 15-20 minutes and record absorbance at 670 nm (end point)***. Do not premix the reagents. They should be added to the well separately. Do not let the plate sit for more than 20 minutes.***

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 220 µl.*

6. **Calculation:** Subtract the standard background from standard OD readings and sample background control readings from sample OD readings. Plot the Standard curve with OD 670 nm (on Y-axis) and NH_4^+ amount in nmol (on X-axis). Obtain the equation from the plot **Y** = a**X** + b, where **Y** is the OD value and **X** is the amount of NH_4^+. Use the equation to calculate amount of NH_4^+ in samples. Calculate the 5'-nucleotidase activity of the test sample as follows:

Detected Activity = B / (Δt X p) (nmol / (min X mg)) = mU/mg

Where: **B** = NH_4^+ amount in sample (nmol).
Δt = reaction time i.e. 20 minutes
p = sample protein content added (mg)

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

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

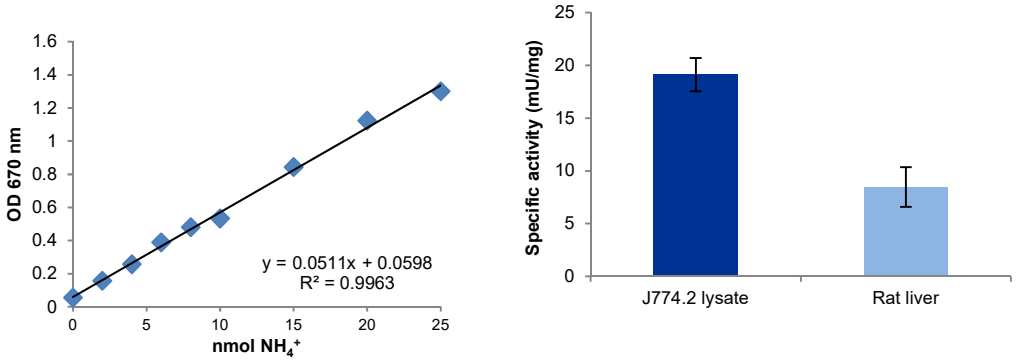


Figure: (a) NH_4^+ standard curve (b) 5'-Nucleotidase specific activity in J774.2 mouse macrophage (60 µg protein) cell line lysate and rat liver tissue lysate (40 µg protein). Assays were performed following kit protocols.

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