Technical Bulletin

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

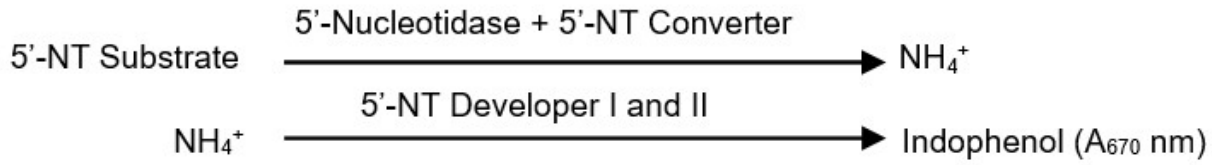
Catalog Number MAK398

Product Description

5′-Nucleotidase (5′-NT), also known at CD73, 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.

The 5′-Nucleotidase (CD73) Activity Assay 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 has a limit of detection of 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 5′-NT activity from the signal from non-specific enzymes. The assay kit also includes 5′-Nucleotidase (5′-NT) enzyme for use as a positive control.

The kit is suitable for the measurement of 5′-Nucleotidase activity in animal tissue lysates (e.g., liver), cell lysates, recombinant enzymes, and purified proteins.



Components

The kit is sufficient for 100 colorimetric assays in 96-well plates.

- 5′-NT Assay Buffer 25 mL
  Catalog Number MAK398A
- 5′-NT Substrate 1 vial
  Catalog Number MAK398B
- 5′-NT Convertor 1 vial
  Catalog Number MAK398C
- 5′-NT Inhibitor 250 µL
  Catalog Number MAK398D
- 5′-NT Stop Solution 500 µL
  Catalog Number MAK398E
- 5′-NT Developer I 8 mL
  Catalog Number MAK398F
- 5′-NT Developer II 4 mL
  Catalog Number MAK398G
- NH_4^+ Standard (100 mM) 100 µL
  Catalog Number MAK398H
- 5′-NT Positive Control 1 vial
  Catalog Number MAK398I

Reagents and Equipment Required but Not Provided

- Pipetting devices and accessories (including multichannel pipettor)
- 96-well clear flat-bottom plate. Cell culture or tissue culture treated plates are **not** recommended.
- Spectrophotometric multiwell plate reader
- Incubator / water bath capable of 37 °C
- Dounce tissue grinder set (Catalog Number D9063 or equivalent)
- Refrigerated microcentrifuge capable of RCF ≥10,000 × *g*
- Bicinchoninic Acid Kit for Protein Determination (Catalog Number BCA1)

Precautions and Disclaimer

For Research Use Only. Not for use in diagnostic procedures. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped on wet ice. Store kit at -20 °C, protected from light.

Preparation Instructions

Briefly centrifuge small vials prior to opening.

5′-NT Buffer: Warm to room temperature prior to use. Chill an appropriate amount of 5′-NT Assay Buffer for use in Sample Preparation.

5′-NT Substrate: Reconstitute vial with 1.1 mL of 5′-NT Assay Buffer. Aliquot and store at -20 °C. Reconstituted substrate is stable for at least two months.

5′-NT Convertor: Reconstitute vial with 220 µL of 5′-NT Assay Buffer before use. Gently pipette up and down to dissolve completely and then centrifuge briefly. Aliquot and store at -80 °C. Keep on ice while in use. Use within two months after reconstitution.

5′-NT Positive Control: Add 22 µL of 5′-NT Assay 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.

Procedure

Sample Preparation

1. Rapidly homogenize tissue (10 mg) or cells (1 x 10^6 cells) with 100 µL of ice cold 5′-NT Assay Buffer and keep on ice for 10 minutes.

2. Centrifuge at 10,000 × *g* for 10 minutes at 4 °C and transfer the supernatant to a fresh tube.
3. Determine the protein concentration using preferred method. Protein determination by BCA is recommended.
4. Protein concentration should range between 1-20 mg/mL. Concentrated samples may be diluted with 5'-NT Assay Buffer.
5. Aliquot and store lysates at -80 °C unless being used immediately.
6. Use 5-20 µL of sample per well. For unknown samples, test several doses to ensure the readings are within the Standard Curve range.
7. Prepare three identical wells for each sample labelled "Sample Background Control" (BC), "Sample" (S) and "Sample + Inhibitor" (SI).
8. For SI well, add 5 µL of 5'-NT Inhibitor in addition to sample.
9. Adjust total volume in each well to 50 µL with 5'-NT Assay Buffer.

Positive Control (PC)

Add 2 µL of 5'-NT Enzyme into desired well, then add 48 µL of 5'-NT Assay Buffer, mix well.

Inhibitor Control (IC)

Add 2 µL of 5'-NT Enzyme into desired well. Add 5 µL of 5'-NT Inhibitor and 43 µL of 5'-NT Assay Buffer, mix well.

Standard Curve Preparation

Prepare a 1 mM Ammonium Standard solution by diluting 10 µL of NH_4^+ Standard (100 mM) with 990 µL of purified water, mix well. Prepare Ammonium Standards according to Table 1. Mix well.

Table 1.
Preparation of Ammonium Standards

Well	1 mM Ammonium Standard	5'-NT Assay Buffer	Ammonium (nmol/ well)
1	0 µL	100 µL	0
2	2 µL	98 µL	2
3	4 µL	96 µL	4
4	6 µL	94 µL	6
5	8 µL	92 µL	8
6	10 µL	90 µL	10
7	15 µL	85 µL	15

Reaction Mix

1. Mix enough reagents for the number of assays to be performed.
   a. For each well containing Sample (S), Sample + Inhibitor (SI), or Positive Control, prepare 50 µL of Sample Reaction Mix according to Table 2, mix well.
   b. For each Sample Background Control well (BC), prepare BC Reaction Mix according to Table 2, mix well.

Table 2.
Reaction Mix Preparation

Reagent	BC Reaction Mix	Sample Reaction Mix
5'- NT Assay Buffer	48 µL	38 µL
5'- NT Converter	2 µL	2 µL
5'- NT Substrate	-	10 µL

Reaction

1. Add the Reaction Mix to wells of the 96-well plate containing the samples and positive control. wells. Add BC Reaction Mix to Sample Background Control wells The volume in every well (S, BC, SI, PC, IC and standards) at this step is 100 µL.
2. Incubate the plate at 37 °C for 20 minutes (if low enzyme activity is observed or expected in samples, incubation time may be increased.)
3. Add 4 µL of stop solution to each well, mix.

4. Add 80 µL of 5′-NT Developer I and 40 µL of 5′-NT Developer II to each well (do not premix the developer reagents, they should be added to the wells separately), mix well. Turbidity upon addition of Developer solutions is normal and will disappear in a few minutes.
5. Incubate at room temperature for 15-20 minutes (do not exceed 20 minutes).

Measurement

Record absorbance at 670 nm (A_{670}) in end point mode.

Results

1. Subtract the 0 Standard A_{670} reading from Standards A_{670} readings.
2. Plot the Standard curve with A_{670} on Y-axis and NH_4^+ amount in nmol on X-axis).
3. Obtain the equation from the plot Y = aX + b, where Y is the A_{670} value and X is the amount of NH_4^+
4. Subtract the Sample Background Control A_{670}readings from Sample A_{670} readings.
5. Use the equation from Step 3 to calculate amount of NH_4^+ in Samples.
6. Calculate the 5′-nucleotidase activity of the test sample as follows:

Detected Activity (mU/mg) =

$$B / [\Delta T \times p]$$

Specific 5′-Nucleotidase Activity in Sample =

Detected Activity in S – Detected Activity in SI

where:

B = NH_4^+ amount in sample (nmol)
ΔT = Reaction time (i.e. 20 minutes per the procedure)
p = Sample protein content added (mg)

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 1.
Typical NH_4^+ Standard Curve

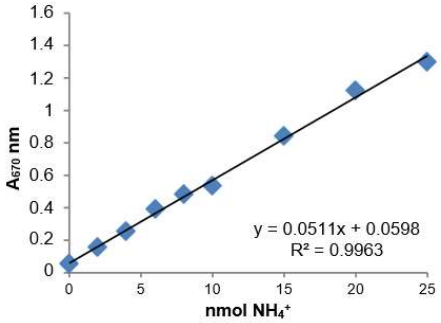
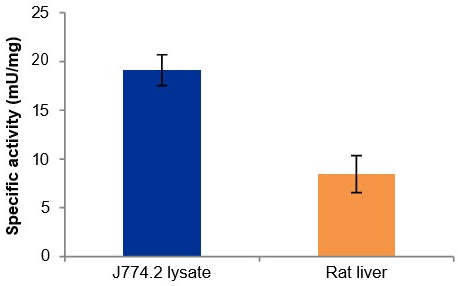


Figure 2.
5′-Nucleotidase specific activity in J774.2 mouse macrophage (60 µg protein) cell line lysate and rat liver tissue lysate (40 µg protein). Assays were performed following the kit protocol.



Frequently Asked Questions

Is the kit specific to CD73? Will it cross react with other enzymes such as CD39?

The substrate used in the kit is specific for CD73 and will not cross-react with CD39. The reaction with the substrate releases ammonia, which is then quantified and enzymes like alkaline phosphatase can also contribute to the generation of the final signal. Hence, the use of 5′-NT inhibitor is recommended to completely inhibit 5′-nucleotidase and distinguish from the signals generated by non-specific enzymes. Thus, the initial reaction is highly specific to CD73 due to substrate specificity.

Notice

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