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ab221820 NMNAT1 Activity Assay Kit (Colorimetric)

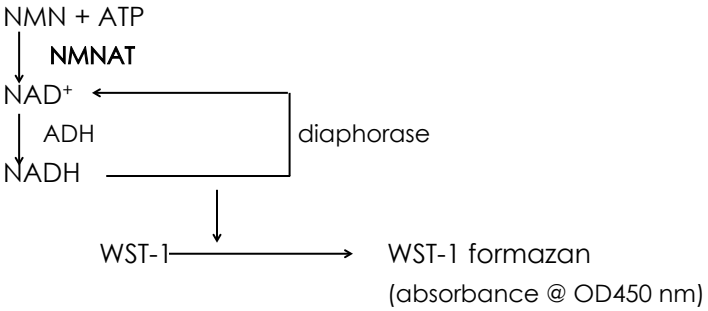
For the rapid, sensitive and accurate measurement of nicotinamide mononucleotide adenylyltransferase (NMNAT) activity in from purified protein or immunopurified cell extracts.

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

Table of Contents

1. Overview

NMNAT1 Activity Assay Kit (Colorimetric) (ab221820) provides a sensitive and robust method to evaluate activators and inhibitors of nicotinamide mononucleotide adenylyltransferase (NMNAT) activity using recombinant NMNAT protein. The assay can also be used to measure activity from endogenous NMNAT immunoprecipitated from cell extracts. The assay is based on a multi-step reaction that converts WST-1 to WST-1 formazan, which can be easily detected at OD 450 nm. As the reaction is not stopped, it is necessary to monitor the absorbance increase of WST-1 formazan at OD450 nm at regular intervals after the reaction is initiated to determine velocity of reaction.



Detection of NMNAT activity can be measured with a One-Step or a Two-Step method. For the One-Step method, the four enzymes involved in the reaction are mixed together. The detection sensitivity of this method is lower than that of the Two-Step method since three coupled reactions occur simultaneously. We recommend performing the One-step assay only when using purified protein.

The Two-Step method is performed by adding the first two enzymes to produce NAD^+, followed by the addition of the rest of the components to form WST-1 formazan by NAD/NADH enzyme cycling reaction.

We recommend performing the Two-Step assay if using immunoprecipitated cell lysates, as the assay procedure allows to check for contamination and interference for NAD^+ present in the sample. Since the assay is based on a NAD^+ detection system, it is

not possible to directly detect NMNAT activity in crude cell extracts, which contain relatively high NAD^+.

For the One-Step method, we describe a procedure to test NMNAT activity using purified enzyme and a separate procedure for screening NMNAT activity inhibitors.

For the Two-Step Method, we describe two different procedures to test NMNAT activity using purified enzyme or to test immunoprecipitated cell lysates. There is also a separate procedure for screening NMNAT activity inhibitors.

Nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1, EC 2.7.7.1) is a central enzyme in NAD+ biosynthesis, transferring the adenylyl moiety of ATP to β-Nicotinamide mononucleotide (NMN) or nicotinic acid mononucleotide (NaMN), resulting in the formation of NAD^+ or NaAD and the release of pyrophosphate. This enzyme could be a potential target for therapeutical applications, because its activity is rather low in tumor cells. It has been shown that NMNAT1 can inhibit recombinant human poly(ADP-ribose) polymerase-1 (PARP-1) by about 35% and completely prevent the formation of branched ADP-ribose polymers.

2. Protocol Summary – One-Step Method

Sample Preparation

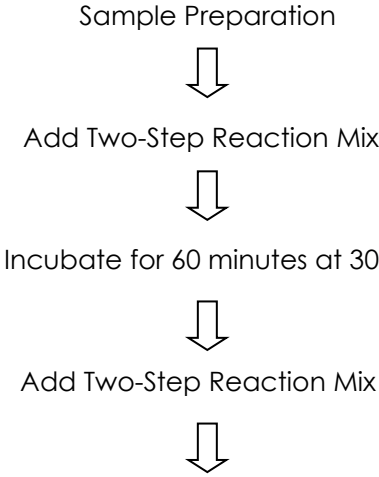
Add One-Step Reaction Mix

Add reaction mix

Incubate for 60 minutes at 30°C and measure absorbance (OD450 nm) in kinetic mode*

**For kinetic mode detection, incubation time given in this summary is for guidance only*

3. Protocol Summary – Two-Step Method



**For kinetic mode detection, incubation time given in this summary is for guidance only*

4. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

5. Storage and Stability

Store kit at -80°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

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

Δ Note: Reconstituted components are stable for 2 months.

6. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

7. Materials Supplied

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
20X NMNAT1 Assay Buffer	1 mL	-80°C	-80°C
WST-1	0.5 mL	-80°C	-80°C
ADH	0.5 mL	-80°C	-80°C
Diaphorase	0.5 mL	-80°C	-80°C
EtOH solution	0.5 mL	-80°C	-80°C
ATP	0.5 mL	-80°C	-80°C
Human NMNAT1	0.5 mL	-80°C	-80°C
NMN	0.5 mL	-80°C	-80°C

8. 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 OD 450 nm – ideally, microplate should be capable of measuring dual wavelengths at 450/540 nm (range 450/550-450/590 nm)
- Double distilled water (ddH_2O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom
- Gallotannin (NMNAT inhibitor) – prepare 0.2 mM stock solution in ddH_2O
- (Optional) β-Nicotinamide adenine dinucleotide (β-NAD): we recommend ab120403 – prepare 1 mM stock solution in ddH_2O

For immunoprecipitation of NMNAT from cell lysates:

- Cell Lysis Buffer: 20 mM Tris (pH 8.0), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1mM DTT and protease inhibitors
- PBS
- Sterile cell scrapper
- Sonicator
- Protein A agarose beads – we recommend Protein A Agarose (ab193254)
- Anti-NMNAT (NMNAT-1 or NMNAT-2) antibody that works in immunoprecipitation

9. Technical Hints

- **This kit is sold based on number of tests. A "test" simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

10. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

10.1 20X NMNAT Assay Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store at -80°C.

10.2 WST-1:

Ready to use as supplied. Thaw on ice before use. Aliquot so that you have enough volume to performed the desired number of assays. Store at -80°C.

10.3 ADH:

Ready to use as supplied. Thaw on ice before use. Aliquot so that you have enough volume to performed the desired number of assays. Avoid freeze/thaw cycles. Store at -80°C.

10.4 Diaphorase:

Ready to use as supplied. Thaw on ice before use. Aliquot so that you have enough volume to performed the desired number of assays. Avoid freeze/thaw cycles. Store at -80°C.

10.5 Ethanol solution:

Ready to use as supplied. Thaw on ice before use. Aliquot so that you have enough volume to performed the desired number of assays. Store at -80°C.

10.6 ATP:

Ready to use as supplied. Thaw on ice before use. Aliquot so that you have enough volume to performed the desired number of assays. Store at -80°C.

10.7 Human NMNAT1 (recombinant, expressed in *E. coli*):

Ready to use as supplied. Thaw on ice before use. Aliquot so that you have enough volume to performed the desired number of assays. Avoid freeze/thaw cycles. Store at -80°C.

10.8 NMN:

Ready to use as supplied. Thaw on ice before use. Aliquot so that you have enough volume to performed the desired number of assays. Store at -80°C.

11. Sample Preparation

- The following cell lysate preparation procedure is intended only as a guideline. The optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the researcher.
- Prior to harvesting cells, prepare ice-cold Cell Lysis Buffer [20 mM Tris (pH 8.0), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1mM DTT + protease inhibitors]. Keep Cell Lysis Buffer on ice while in use.

11.1 Purified protein:

No sample preparation is required. If necessary, protein can be diluted in 1X NMNAT buffer.

11.2 Inhibitor Screening compounds

Dissolve test inhibitors to a 20X solution using appropriate solvent.

11.3 Immunoprecipitated cell lysates:

11.3.1 Grow your cells of interest in the appropriate cell culture media.

Δ Note: If desired, treat cells for desired time by adding fresh culture media containing test compounds of interest.

11.3.2 Aspirate/remove media from cells. Rinse cells once with ice-cold PBS.

11.3.3 Add 0.5 mL of ice-cold Cell Lysis Buffer to each 10-cm dish plate and incubate the plate on ice for 5 minutes.

Δ Note: adapt volume of Cell Lysis Buffer if using cell culture vessels.

11.3.4 Scrape cell off the plate with a cell scraper and transfer to microcentrifuge tubes. Keep on ice.

11.3.5 Sonicate 4 times for 5 seconds each, on ice.

11.3.6 Centrifuge for 10 minutes at 4°C at 13,000 *g* in a cold microcentrifuge to remove any insoluble material.

11.3.7 Transfer supernatant to a new tube. Keep on ice.

Δ Note: cell lysate can be stored at -80°C.

11.3.8 Take 250 µL cell lysate and add 40 µL protein A Agarose beads (50% slurry). Incubate with gentle rocking for 1-3 hours at 4°C for pre-clearance

11.3.9 Centrifuge sample for 30 second at 4°C at 13,000 *g* in a cold microcentrifuge. Take supernatant and transfer to a new tube.

11.3.10 Add 1-2 µg of specific antibody against NMNAT family members (anti-NMNAT1 or anti-NMNAT2) that can be used for immunoprecipitation, and incubate with gentle rocking for 2 h at RT/overnight at 4°C.

11.3.11 Add 20 µL protein A agarose beads (50% bead slurry) and incubate sample with gentle rocking for 1-3 hours at 4°C.

11.3.12 Centrifuge sample for 10 second at 4°C at 13,000 *g* in a cold microcentrifuge. Discard supernatant very carefully, making sure you don't discard any beads.

11.3.13 Was beads twice with 500 µL ice-cold Cell Lysis Buffer. Keep tube on ice.

11.3.14 Wash beads twice with 500 µL ice-cold 1X NMNAT Assay Buffer. Keep tube on ice.

11.3.15 Resuspend beads in 20 µL ice-cold 1X NMNAT Assay Buffer. Keep sample on ice.

12. Assay Procedure – One-step Activity Assay

- We recommend that you assay all controls and samples in duplicate.
- Prepare all reagents and samples as directed in the previous sections.
- Use this protocol to measure NMNAT activity from purified protein only.
- For unknown samples, we recommend testing different volumes.

Δ Note: avoid mixing any reagents containing thiol groups (such as DTT or reduced glutathione) or alkyl amines in the sample as they will interfere with the assay.

12.1 One-Step Activity Reaction mix:

12.1.1 Prepare 60 µL of Reaction Mix for each reaction. Prepare a master mix to ensure consistency.

Component	One-Step Reaction Mix (µL)
20X NMNAT Assay Buffer	5
WST-1	5
ADH	5
Diaphorase	5
Ethanol Solution	5
ATP	5
NMN	5
ddH_2O	25

Δ Note: keep reaction mix on ice and use within 30 minutes of preparation. Discard any unused mixture after use.

12.2 Reaction wells set up:

- Sample wells = 2-5 µL protein (adjust volume to 40 µL/well with ddH_2O).
- No enzyme control = 5 µL 1X NMNAT Assay Buffer + 35 µL ddH_2O.
- Positive control = 5 µL human NMNAT + 35 µL ddH_2O.

12.3 NMNAT reaction:

12.3.1 Initiate reaction by adding 60 µL of Reaction Mix into each reaction well. Mix thoroughly by pipetting up and down.

The table below summarizes the reaction set up (Step 12.2 + 12.3):

Component	Sample well (µL)	No Enzyme control (µL)	Positive control (µL)
Sample protein test	2-5	-	-
1X NMNAT Assay Buffer	-	5	-
Human NMNAT1 (control)	-	-	5
ddH_2O	35-38	35	35
One-Step Reaction Mix	60	60	60
Total reaction volume	100	100	100

12.4 Measurement:

12.4.1 Measure output at OD 450 nm on a microplate reader in kinetic mode every 5 minutes for at least 60 minutes at 30°C protected from light.

Δ Note: Measure activity while reaction velocity remains constant, Incubation reaction time will depend on the NMNAT activity in the samples. We recommend measuring OD in a kinetic mode, and choosing two time points (T1 and T2) to calculate the NMNAT activity of the samples.

13. Assay Procedure – Two-step Activity Assay for purified NMNAT protein

- We recommend that you assay all controls and samples in duplicate.
- Prepare all reagents and samples as directed in the previous sections.
- This protocol can be used to measure NMNAT activity from purified NMNAT protein as it is more sensitive than the One-step method.
- For unknown samples, we recommend testing different volumes.

Δ Note: avoid mixing any reagents containing thiol groups (such as DTT or reduced glutathione) or alkyl amines in the sample as they will interfere with the assay.

13.1 Two-Step Activity Reaction mix:

13.1.1 Prepare 60 µL of Two-Step Reaction Mix I for each reaction. Prepare a master mix to ensure consistency.

Component	Two-Step Reaction Mix I (µL)
20X NMNAT Assay Buffer	5
ATP	5
NMN	5
ddH_2O	45

13.1.2 Prepare 20 µL of Two-Step Reaction Mix II for each reaction. Prepare a master mix to ensure consistency.

Component	Two-Step Reaction Mix II (µL)
WST-1	5
ADH	5
Diaphorase	5
Ethanol Solution	5

Δ Note: Keep Reaction mix I and II on ice and use within 30 minutes of preparation. Discard any unused mixtures after use.

13.2 Reaction wells set up:

- Sample wells = 2-5 µL sample (adjust volume to 20 µL/well with ddH_2O).
- No enzyme control = 5 µL 1X NMNAT Assay Buffer + 15 µL ddH_2O.
- Positive control = 5 µL human NMNAT + 15 µL ddH_2O.

13.3 1st Reaction (conversion of nicotinamide to NAD^+):

13.3.1 Initiate reaction by adding 60 µL of Two-step Reaction Mix I into each reaction well and mixing thoroughly by pipetting up and down.

The table below summarizes the reaction set up (Step 13.2 + 13.3):

Component	Sample well (µL)	No Enzyme control (µL)	Positive control (µL)
Sample test - PROTEIN	2-5	-	-
1X NMNAT Assay Buffer	-	5	-
Human NMNAT (control)	-	-	5
ddH_2O	15-18	15	15
Two-Step Reaction Mix I	60	60	60
Total reaction volume	80	80	80

13.3.2 Incubate plate at 30°C for 60 minutes.

13.4 2nd Reaction (measurement of generated NAD^+):

13.4.1 Initiate reaction by adding 20 µL Two-Step Reaction Mix II into each reaction well. Mix thoroughly up and down.

13.5 Measurement:

13.5.1 Measure output at OD 450 nm on a microplate reader in kinetic mode every 5 minutes for at least 30 minutes at 30°C protected from light.

Δ Note: Measure activity while the reaction velocity remains constant. Incubation time depends on the NMNAT activity in the samples. We recommend measuring OD in a kinetic mode, and choosing two time points (T1 and T2) to calculate the NMNAT activity of the samples.

14. Assay Procedure – Two-step Activity Assay for immunoprecipitated cell lysates

- We recommend that you assay all controls and samples in duplicate.
- Prepare all reagents and samples as directed in the previous sections.
- This protocol is recommended for measuring NMNAT activity from immunoprecipitated cell lysates.
- For unknown samples, we recommend testing different volumes.

Δ Note: avoid mixing any reagents containing thiol groups (such as DTT or reduced glutathione) or alkyl amines in the sample as they will interfere with the assay.

14.1 Two-Step Activity Reaction mix:

14.1.1 Prepare 60 µL of Two-Step Reaction Mix I for each reaction and 60 µL of No NMN Reaction Mix. Prepare a master mix to ensure consistency.

Δ Note: No NMN Reaction mix is used as background control to ensure NMNAT activity is measured correctly.

Component	Two-Step Reaction Mix I (µL)	No NMN Reaction Mix (µL)
20X NMNAT Assay Buffer	5	5
ATP	5	5
NMN	5	-
ddH_2O	45	50

14.1.2 Prepare 20 µL of Two-Step Reaction Mix II for each reaction. Prepare a master mix to ensure consistency.

Component	Two-Step Reaction Mix II (µL)
WST-1	5
ADH	5
Diaphorase	5
Ethanol Solution	5

Δ Note: Keep Reaction mix I and II on ice and use within 30 minutes of preparation. Discard any unused mixtures after use.

14.2 Reaction wells set up:

- Sample wells = 2-5 µL sample (adjust volume to 20 µL/well with ddH_2O).
- No NMN control = 2-5 µL sample (adjust volume to 20 µL/well with ddH_2O).
- Positive control = 5 µL human NMNAT + 15 µL ddH_2O.
- No enzyme control = 20 µL ddH_2O.

14.3 Set up 1st Reaction (conversion of nicotinamide to NAD^+):

14.3.1 Initiate reaction by adding 60 µL of Two-Step Reaction Mix I into each sample, no enzyme control and positive control reaction wells. Mix thoroughly by pipetting up and down.

14.3.2 Initiate reaction by adding 60 µL of No NMN Reaction Mix into the No NMN control well. Mix thoroughly by pipetting up and down.

The table below summarizes the reaction set up (Step 14.2 + 14.3):

Component	Sample well (µL)	No NMN control (µL)	Positive control (µL)	No Enzyme control (µL)
Sample test - LYSATES	2-5	2-5	-	-
Human NMNAT (control)	-	-	5	-
ddH_2O	15-18	15-18	15	20
Two-Step Reaction Mix I	60	-	60	60
No NMN Reaction Mix	-	60	60	60

Total reaction volume	80	80	80	80

14.3.3 Incubate plate at 30°C for 30 minutes.

14.4 2nd Reaction (measurement of generated NAD^+):

14.4.1 Initiate reaction by adding 20 µL Two-Step Reaction Mix II into each reaction well. Mix thoroughly by pipetting up and down.

14.5 Measurement:

14.5.1 Measure output at OD 450 nm on a microplate reader in kinetic mode, every 5 minutes, for at least 30-60 minutes at 30°C protected from light.

Δ Note: Measure activity while the reaction velocity remains constant. Incubation time depends on the NMNAT activity in the samples. We recommend measuring OD in a kinetic mode, and choosing two time points (T1 and T2) to calculate the NMNAT activity of the samples.

14.6 Optional – NAD^+ contamination control step:

Since NAD^+ level in cells is relatively high, NAD^+ might be present in the purified NMNAT sample even after immunoprecipitation. This contamination can cause a false positive result by initiating the NAD^+/NADH enzyme cycling reaction. If you suspect this might happen in your assay, we recommend you perform this step to check for NAD^+ contamination.

Alternatively, you can use NAD/NADH Assay Kit II (Colorimetric) (ab221821) to detect NAD/NADH contamination.

14.6.1 Prepare a 10 µM NAD^+ solution from a 1 mM stock solution (not provided) in ddH_2O immediately prior to use.

14.6.2 Set up the control reaction as displayed in the table below:

Component	Test compound well (µL)	NAD^+ control well (µL)
Sample test	2-5	-
NAD^+ (10 µM)	-	5
ddH_2O	75-78	75

14.6.3 Add 20 µL Two-Step Reaction Mix II (Step 14.1.2) into each reaction well and mix thoroughly.

14.6.4 Measure output at OD 450 nm on a microplate reader in kinetic mode, every 5 minutes, for at least 60 minutes at 30°C protected from light.

Δ Note: Measure activity while the reaction velocity remains constant.

If there is contamination of NAD^+ in your sample, OD450 nm signal will increase at the same rate as the one the NAD^+ control well.

15. Assay Procedure – One-step Inhibitor Screening

- We recommend that you assay all controls and samples in duplicate.
- Prepare all reagents and samples as directed in the previous sections.
- For unknown samples, we recommend testing different volumes.
- This protocol can be used for screening compounds that have an inhibitory effect on NMNAT activity. Gallotannin, a potent inhibitor of NMNAT activity, can be used as inhibitor control but it is not provided with the kit.

15.1 Inhibitor control stock preparation:

15.1.1 Prepare a 0.2 mM Stock solution of Gallotannin in ddH_2O immediately prior to use.

15.2 One-Step Activity Reaction mix:

15.2.1 Prepare 60 µL of One-Step Reaction Mix for each reaction. Prepare a master mix to ensure consistency.

Component	One-Step Reaction Mix (µL)
20X NMNAT Assay Buffer	5
WST-1	5
ADH	5
Diaphorase	5
Ethanol Solution	5
ATP	5
NMN	5
ddH_2O	25

Δ Note: keep reaction mix on ice and use within 30 minutes of preparation. Discard any unused mixture after use.

15.3 Reaction wells set up:

- Inhibitor test sample wells = 1-5 µL test inhibitor (20X stock solution) + 5 µL NMNAT (adjust volume to 35 µL/well with ddH_2O).
- Solvent control well = 5 µL solvent + 5 µL NMNAT + 30 µL ddH_2O.
- Inhibitor control well = 5 µL NMNAT + 5 µL Gallotannin + 30 µL ddH_2O.

15.4 NMNAT reaction:

15.4.1 Initiate reaction by adding 60 µL of Two-step Reaction Mix I into each reaction well and mixing thoroughly by pipetting up and down.

The table below summarizes the reaction set up (Step 15.2 + 15.3):

Component	Sample well (µL)	Solvent control (µL)	Inhibitor control (µL)
20X Inhibitor test	1-5	-	-
Solvent	-	5	-
Gallotannin (0.2 mM)			5
Human NMNAT (control)	5	5	5
ddH_2O	30-34	30	30
One-Step Reaction Mix	60	60	60
Total reaction volume	100	100	100

15.5 Measurement:

15.5.1 Measure output at OD 450 nm on a microplate reader in kinetic mode, every 5 minutes, for at least 60 minutes at 30°C protected from light.

Δ Note: Measure activity while the reaction velocity remains constant. Incubation time will depend on the NMNAT activity in the samples. We recommend measuring OD in a kinetic mode, and choosing two time points (T1 and T2) to calculate the NMNAT activity of the samples.

15.6 Optional – Inhibitory control assay step:

If inhibitor compound tested has an inhibitory effect on one of the other two enzymes involved in the reaction (ADH or diaphorase), the signal will be reduced. If you suspect this might happen in your assay, we recommend you perform this step to check for unspecific activity assay coupled with NAD^+/NADH enzyme cycling reaction.

15.6.1 Prepare a 10 µM NAD^+ solution from a 1 mM stock solution (not provided) in ddH_2O immediately prior to use.

15.6.2 Set up the control reaction as displayed in the table below:

Component	Test compound well (µL)	Solvent control well (µL)
20X Inhibitor test	1-5	-
Solvent	-	5
NAD^+ (10 µM)	5	5
ddH_2O	70-74	70
WST-1	5	5
ADH	5	5
Diaphorase	5	5
Ethanol Solution	5	5

15.6.3 Measure output at OD 450 nm on a microplate reader in kinetic mode, every 5 minutes, for at least 60 minutes at 30°C protected from light.

Δ Note: Measure activity while reaction velocity remains constant.

If there is an inhibitory effect of your test inhibitor compound on the unspecific activity (ADH or diaphorase) coupled to NAD^+/NADH cycling reaction, OD450 nm signal will not increase in the Test compound well.

16. Assay Procedure – Two-step Inhibitor Screening

- We recommend that you assay all controls and samples in duplicate.
- Prepare all reagents and samples as directed in the previous sections.
- For unknown samples, we recommend testing different volumes.
- This protocol can be used for screening compounds that have an inhibitory effect on NMNAT activity as it is more sensitive than the One-step method. Gallotannin, a potent inhibitor of NMNAT activity, can be used as inhibitor control but it is not provided with the kit.

16.1 Two-Step Activity Reaction mix:

16.1.1 Prepare 60 µL of Two-Step Reaction Mix I for each reaction. Prepare a master mix to ensure consistency.

Component	Two-Step Reaction Mix I (µL)
20X NMNAT Assay Buffer	5
ATP	5
NMN	5
ddH_2O	45

16.1.2 Prepare 20 µL of Two-Step Reaction Mix II for each reaction. Prepare a master mix to ensure consistency.

Component	Two-Step Reaction Mix II (µL)
WST-1	5
ADH	5
Diaphorase	5
Ethanol Solution	5

Δ Note: Keep Reaction mix I and II on ice and use within 30 minutes of preparation. Discard any unused mixtures after use.

16.2 Reaction wells set up:

- Inhibitor test sample wells = 1-5 µL test inhibitor (20X stock solution) + 5 µL NMNAT (adjust volume to 15 µL/well with ddH_2O).
- Solvent control well = 5 µL solvent + 5 µL NMNAT + 10 µL ddH_2O.
- Inhibitor control well = 5 µL NMNAT + 5 µL Gallotannin + 10 µL ddH_2O.

16.3 1st Reaction (conversion of nicotinamide to NAD^+):

16.3.1 Initiate reaction by adding 60 µL of Two-step Reaction Mix I into each reaction well and mixing thoroughly by pipetting up and down.

The table below summarizes the reaction set up (Step 16.2 + 16.3):

Component	Sample well (µL)	Solvent control (µL)	Inhibitor control (µL)
20X Inhibitor test	1-5	-	-
Solvent	-	5	-
Gallotannin (0.2 mM)			5
Human NMNAT (control)	5	5	5
ddH_2O	10-14	10	10
Two-Step Reaction Mix I	60	60	60
Total reaction volume	100	100	100

16.3.2 Incubate plate at 30°C for 60 minutes.

16.4 2nd Reaction (measurement of generated NAD^+):

16.4.1 Initiate reaction by adding 20 µL Two-Step Reaction Mix II into each reaction well. Mix thoroughly up and down.

16.5 Measurement:

16.5.1 Measure output at OD 450 nm on a microplate reader in kinetic mode, every 5 minutes, for at least 30 minutes at 30°C protected from light.

Δ Note: Measure activity while the reaction velocity remains constant. Incubation time depends on the NMNAT activity in the samples. We recommend measuring OD in a kinetic mode, and

choosing two time points (T1 and T2) to calculate the NMNAT activity of the samples.

16.6 Optional – Inhibitory control assay step:

If inhibitor compound tested has an inhibitory effect on one of the other two enzymes involved in the reaction (ADH or diaphorase), the signal will be reduced. If you suspect this might happen in your assay, we recommend you perform this step to check for unspecific activity assay coupled with NAD^+/NADH enzyme cycling reaction.

16.6.1 Prepare a 10 µM NAD^+ solution from a 1 mM stock solution (not provided) in ddH_2O immediately prior to use.

16.6.2 Set up the control reaction as displayed in the table below:

Component	Test compound well (µL)	Solvent control well (µL)
20X Inhibitor test	1-5	-
Solvent	-	5
NAD^+ (10 µM)	5	5
ddH_2O	70-74	70

16.6.3 Add 20 µL Two-Step Reaction Mix II (Step 16.1.2) into each reaction well and mix thoroughly.

16.6.4 Measure output at OD 450 nm on a microplate reader in kinetic mode, every 5 minutes, for at least 60 minutes at 30°C protected from light.

Δ Note: Measure activity while reaction velocity remains constant.

If there is an inhibitory effect of your test inhibitor compound on the unspecific activity (ADH or diaphorase) coupled to NAD^+/NADH cycling reaction, OD450 nm signal will not increase in the Test compound well.

17. Calculations

- Measure activity while the reaction velocity remains constant.
- Use only the linear rate for calculation.

MEASUREMENT OF NMNAT ACTIVITY IN THE SAMPLE:

- Determine the corrected change in absorbance intensity for each sample well by subtracting the OD value of the "No enzyme control" well at all reaction time points.
- For all sample and other control reaction wells, choose two time points (T1 and T2) in the linear phase of the reaction progress curves and obtain the corresponding OD values at those points (OD1 and OD2).
- NMNAT activity in the sample is shown as reaction rate (ΔOD/min):

$$\text{Activity } [\Delta OD450nm] = (A2 - A1) / (T2 - T1)$$

INHIBITOR COMPOUND SCREENING:

- Determine the corrected change in absorbance intensity for each sample well by subtracting the OD value of the "Solvent control" well at all reaction time points.
- For all sample and other control reaction wells, choose two time points (T1 and T2) in the linear phase of the reaction progress curves and obtain the corresponding OD values at those points (OD1 and OD2).
- NMNAT activity in the sample is shown as reaction rate (ΔOD/min):

$$\text{Activity } [\Delta OD450nm] = (A2 - A1) / (T2 - T1)$$

If your Test Compound causes an inhibitory effect on NAMPT enzymatic activity, the OD450 nm is lower than that measured for the "Solvent Control" assay.

18. Typical Data

Data provided for **demonstration purposes only**.

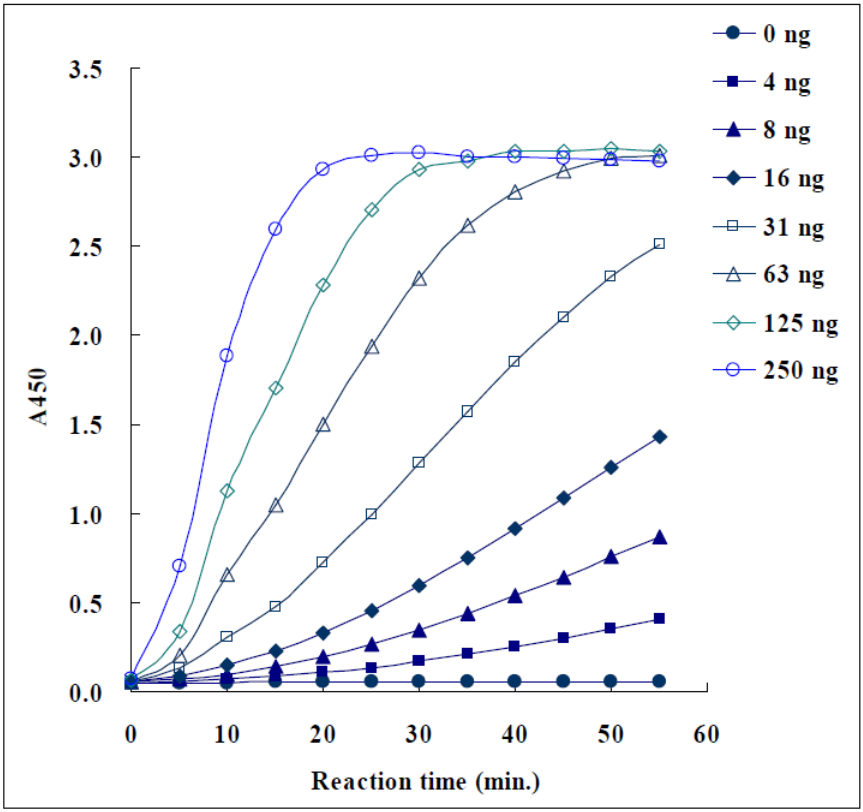


Figure 1. Time course kinetic curve of recombinant NMNAT activity using One-Step Assay Method (Section 12).

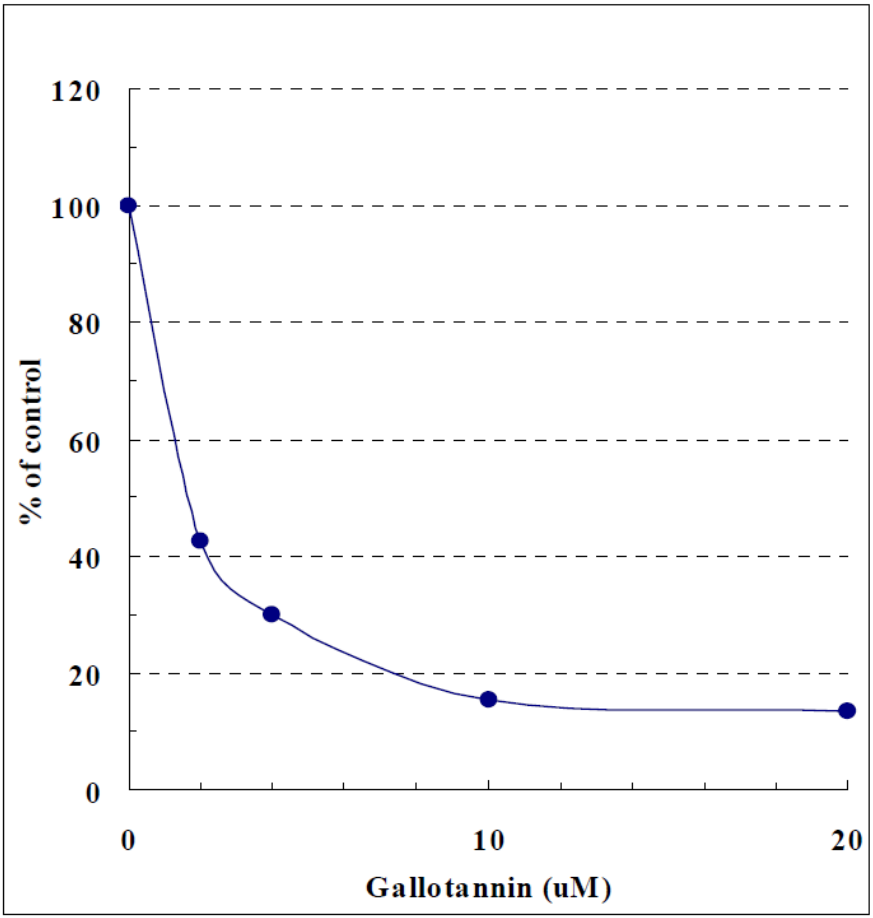


Figure 2. Inhibitory effect of Gallotannin on recombinant NMNAT activity using One-Step Assay Method (Section 15).

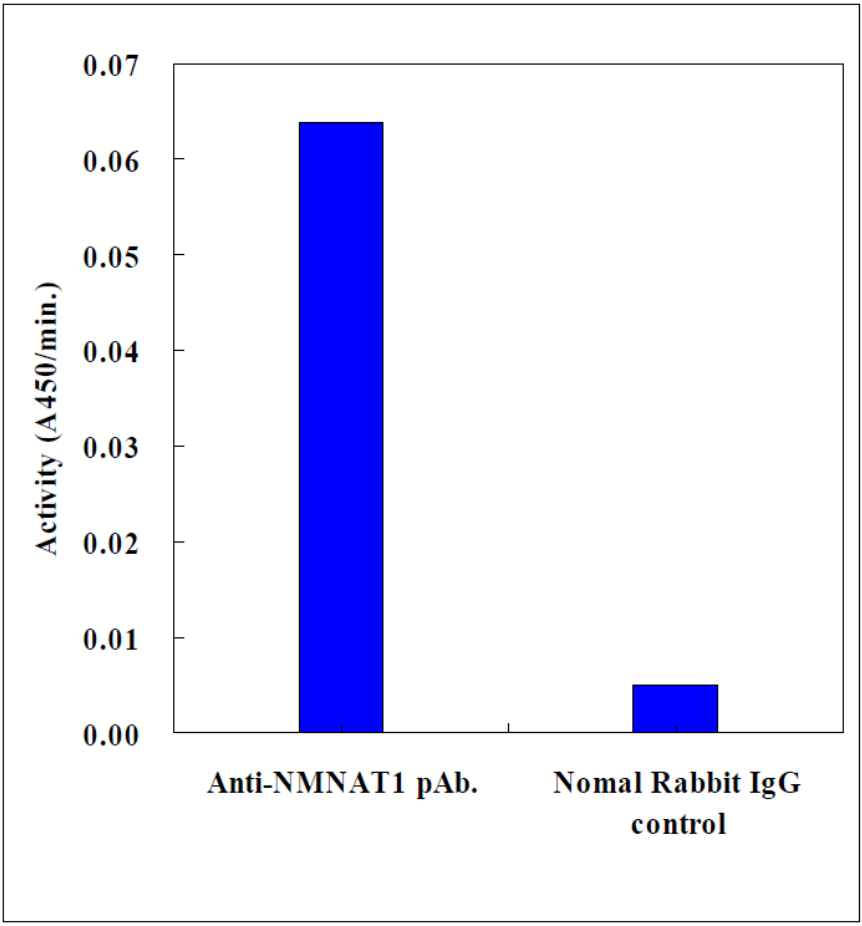


Figure 4. Measurement of NMNAT activity on immunoprecipitated cell lysates from Raji cell extracts (Section 14). Normal rabbit IgG control shows background activity compard to anti-NMNAT1 immunoprecipitate.

19. Troubleshooting

Problem	Reason	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at assay temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different microplate	Colorimetric: clear plates Fluorometric: black wells/clear bottom plates Luminometric: white wells/clear bottom plates
Sample with erratic readings	Samples not clarified completely	Repeat immunoprecipitation
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

20. Interferences

These chemical or biological materials will cause interference in this assay causing compromised results or complete failure:

- Reagents containing thiol groups such as DTT or reduced glutathione
- Reagents containing alkyl amine
- Reagents that inhibit ADH or diaphorase activity

21. Notes

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