

ab204705

**N-Acetylglucosaminidase
(beta-NAG) Activity
Assay Kit (Colorimetric)**

Instructions for Use

For rapid, sensitive and accurate measuring of beta-NAG (N-Acetylglucosaminidase) activity.

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

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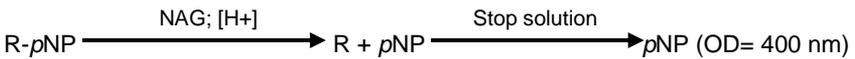
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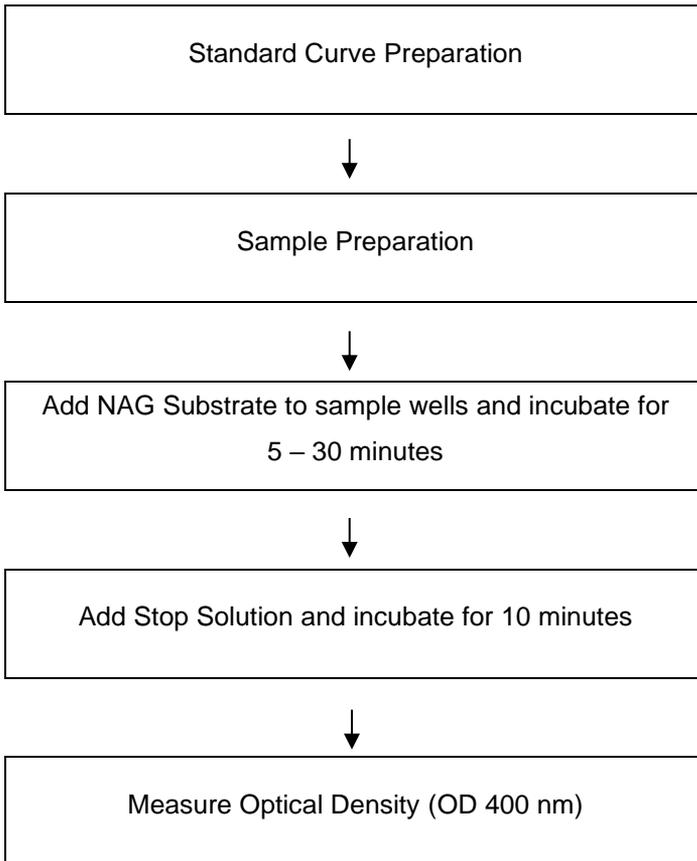
1. BACKGROUND

N-Acetylglucosaminidase (beta-NAG) Activity Assay Kit (Colorimetric) (ab204705) provides a simple and sensitive method for monitoring NAG enzymatic activity. In this assay, NAG uses a synthetic p-nitrophenol derivative (R-pNP) as a NAG substrate and releases pNP which can be measured at absorbance (OD 400 nm). The assay can detect as low as 50 μ U of NAG activity in a variety of samples.



β -N-Acetylglucosaminidase (NAG, EC 3.2.1.52) is a lysosomal enzyme that is expressed in various tissues, including kidney, liver and lungs. NAG can cleave N-acetyl-glucosamine, a monosaccharide derivative of glucose. Its concentration in urine is minimal due to its inability to cross the glomerular basal membrane. Increased concentration of NAG in urine indicates renal tubular cell breakdown. Acute Kidney Injury (AKI) is the sudden loss of kidney functions, causing electrolyte imbalance, and retention of urea and other nitrogenous products. NAG has become one of the most studied and used biomarkers for the detection and diagnosis of AKI.

2. ASSAY SUMMARY



3. 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. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at -20°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 Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

5. 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.

6. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
NAG Assay Buffer	35 mL	-20°C	-20°C
NAG Substrate	6 mL	-20°C	-20°C
Nitrophenol Standard	400 µL	-20°C	-20°C
NAG Stop Solution	3 mL	-20°C	-20°C
NAG Positive Control	1 vial	-20°C	-20°C

Note: pNP is light sensitive and should be kept away from light.

*Some may receive Nitrophenol Standard with a component size of 100 µL until old lots last.

7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- PBS
- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric microplate reader – equipped with filter for OD 400 nm
- 96 well plate: clear plate with flat bottom
- Heat block or water bath
- Dounce homogenizer or pestle (if using tissue)
- Optional: 10 kD Spin column (ab93349) – for colored serum samples.

8. 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.
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.
 - 9.1 **NAG Assay Buffer:** Bring to room temperature (RT) before use. Store at -20°C.
 - 9.2 **NAG Substrate:** Light sensitive. Store at -20°C. Once opened, use within two months. Bring to RT before use. Mix well. If precipitation is observed, sonicate the contents in a water bath sonicator (interval: 2 min). Repeat if necessary.
 - 9.3 **Nitrophenol Standard:** Ready to use. Light sensitive. Bring to RT before use. Store at -20°C. Once opened, use within two months.
 - 9.4 **NAG Stop Solution:** Bring to RT before use. Store at -20 °C. Once opened, use within two months.
 - 9.5 **NAG Positive Control:** Reconstitute with 40 µl NAG Assay Buffer. Mix well. Store at -20°C. Use within two months.

10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and cannot be stored for future use.

10.1 Add 0, 2, 4, 6, 8, and 10 μL of Nitrophenol Standard into a series of wells in a 96-well plate to generate 0, 4, 8, 12, 16 and 20 nmol of pNP/well respectively. Adjust the volume to 125 μL /well with NAG Assay Buffer.

Standard #	Volume of Standard (μL)	Assay Buffer (μL)	Final volume standard in well (μL)	End amount pNP in well (nmol/well)
1	0	375	125	0
2	6	369	125	4
3	12	363	125	8
4	18	357	125	12
5	24	351	125	16
6	30	345	125	20

Each dilution has enough amount of standard to set up duplicate readings (2 x 125 μL).

11. 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. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples 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.

11.1 Cell (adherent or suspension) samples:

- 11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 1×10^6 cells).
- 11.1.2 Wash cells with cold PBS.
- 11.1.3 Resuspend cells in 100 – 200 μL of ice cold NAG Assay Buffer.
- 11.1.4 Homogenize cells quickly by pipetting up and down a few times.
- 11.1.5 Centrifuge samples at $10,000 \times g$ for 3 minutes at 4°C using a cold microcentrifuge to remove any insoluble material.
- 11.1.6 Collect supernatant and transfer to a clean tube.

11.2 Tissue samples:

- 11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 10 – 20 mg).
- 11.2.2 Wash cells with cold PBS.
- 11.2.3 Resuspend tissue in 100 – 200 μL of ice cold NAG Assay Buffer.
- 11.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.

11.2.5 Centrifuge samples at 10,000 x *g* for 3 minutes at 4°C using a cold microcentrifuge to remove any insoluble material.

11.2.6 Collect supernatant and transfer to a clean tube.

11.3 **Urine samples:**

Urine samples can be tested directly by adding sample to the microplate wells. If precipitation is observed, centrifuge urine samples at 10,000 x *g*, 4°C for 3 minutes and collect supernatant.

11.4 **Serum samples:**

Serum samples can be tested directly by adding sample to the microplate wells.

Yellow and pink chromophores might be present in serum samples, absorbing light around OD 400 nm which can skew data. If this is the case, we recommend to test different background control wells by each different color.

Additionally, you can pass the serum samples through a 10 kD spin column (ab93349), discard flow-through (to eliminate any small molecules that are chromophores) and collect concentrated fraction. Sample can be diluted prior centrifugation.

NOTE: *We suggest using different volumes of sample to ensure readings are within the Standard Curve range.*

12. ASSAY PROCEDURE and DETECTION

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend to assay all standards, controls and samples in duplicate.
- Different sample colors can affect the readout. It is not correct to compare samples of different color unless each color has its own control/blank. We recommend to set up a color match control/blank sample.

12.1 Set up Reaction wells:

- Standard wells = 125 μ L standard dilutions.
- Sample wells = 1 – 70 μ L samples (adjust volume to 70 μ L/well with NAG Assay Buffer).
- Positive control = 1 – 5 μ L (adjust volume to 70 μ L/well with NAG Assay Buffer).
- Sample background control wells= 1 – 70 μ L samples (adjust volume to 70 μ L/well with NAG Assay Buffer).

NOTE: *If serum samples have different colors, we recommend to set up sample background control wells for each different colors.*

- 12.2 Add 55 μ L of NAG Substrate into each sample and positive control well.
- 12.3 Add 55 μ L of NAG Assay Buffer to sample background control wells.
- 12.4 Mix and incubate at 37°C for 5 – 30 minutes.
- 12.5 Add 25 μ L of NAG Stop Solution into all wells including Samples, Positive Control, Background Control and Standards.

NOTE: Failure to add stop solution could lead to low OD values

- 12.6 Mix and incubate at 37°C for 10 minutes protected from light.

12.7 Measure output at OD 400nm on a microplate reader.

NOTE: *Incubation time depends on the NAG enzymatic activity in samples. Longer incubation time may be required for samples having low NAG activity.*

13. CALCULATIONS

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
 - 13.1 Average the duplicate reading for each standard and sample.
 - 13.2 If the sample background control is significant, then subtract the sample background control from positive control and sample reading.

For serum samples with different colors, subtract the appropriate matched control. In this case, experimental values will be relative and will need to be normalized.
 - 13.3 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.
 - 13.4 Plot the corrected absorbance values for each standard as a function of the final amount of pNP generated by NAG during the reaction time.
 - 13.5 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).

- 13.6 NAG activity (nmol/min/ml or mU/ml or U/L) in the test samples is calculated as:

$$\text{NAG activity} = \left(\frac{B}{T * V} \right) * D$$

Where:

B = Amount of pNP in the sample well from Standard Curve (nmol)

T = Incubation time for substrate hydrolysis (5 – 30 minutes.)

V = Sample volume added into the reaction well (mL)

D = Sample dilution factor

Sample NAG activity can also be expressed as mU/mg of protein or mU/mg creatinine (urine).

Unit Definition:

1 Unit N-acetylglucosaminidase activity = amount of enzyme that generates 1.0 μmol of pNP per minute at pH 4.2 at 37°C.

14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

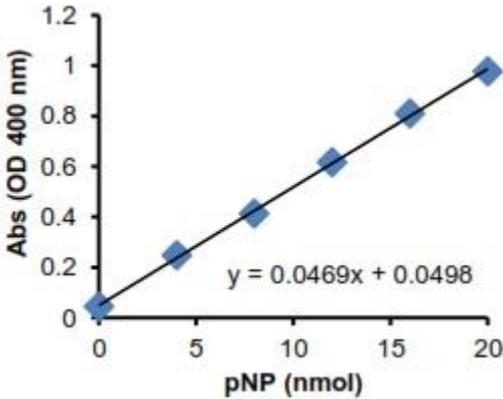


Figure 1. Typical pNP Standard calibration curve

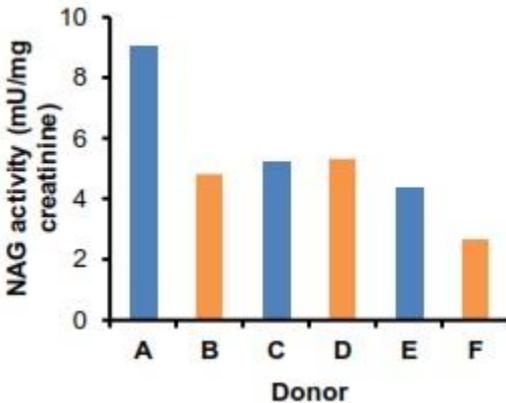


Figure 2. Measurement of NAG activity in human urine from different donors. Undiluted samples (70 µL) were incubated for 30 minutes with NAG substrate.

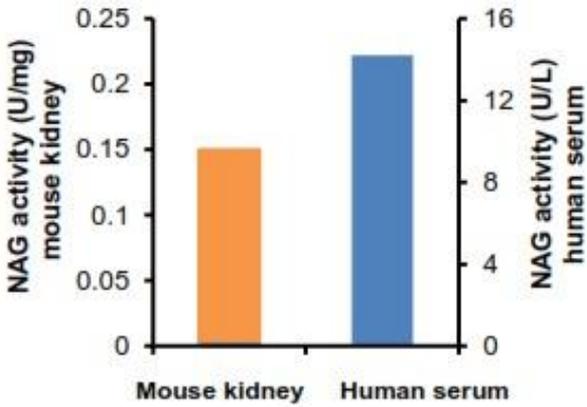


Figure 3. Measurement of NAG activity in mouse kidney (10 μ g) and human serum (20 μ L). Samples were incubated for 30 minutes with NAG substrate.

15. QUICK ASSAY PROCEDURE

NOTE: *This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.*

- Prepare standard, positive control and sample background control; get equipment ready.
- Prepare standard curve.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Set up plate for standard (125 μL), samples (70 μL), positive control (70 μL) and sample background wells (70 μL).
- Add 55 μL of NAG Substrate to the positive control and sample wells.
- Add 55 μL of NAG Assay Buffer to sample background control wells
- Incubate plate at 37°C 5 – 30 minutes.
- Add 25 μL of NAG Stop Solution into all wells.
- Incubate plate at 37°C 10 minutes protected from light.
- Measure plate at OD 400 nm.

16. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use Deproteinizing Sample Kit – TCA (ab204708) or PCA precipitation protocol for deproteinization
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	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
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

RESOURCES

Problem	Cause	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 μ L) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions on protocol
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

17. NOTES

Technical Support

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