ab204725

Aminohippuric acid Assay Kit (Colorimetric)

Instructions for Use

For rapid, sensitive and accurate measuring of Aminohippuric acid in various biological samples.

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

Version 3 Last Updated 18 August 2015

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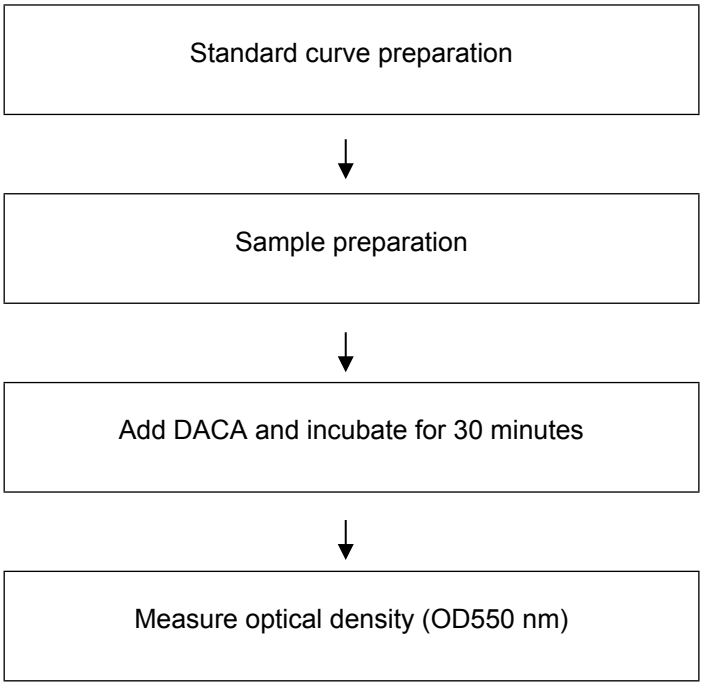
1. BACKGROUND

Aminohippuric acid Assay Kit (ab204725) is based on a 96-well format in which p-Aminohippuric acid (PAH) present in the sample reacts with Dimethylaminocinnamaldehyde (DACA), which gives a strongly colored derivative at OD = 550 nm. The amounts of PAH in urine and plasma can easily be quantified.

p-Aminohippuric acid (PAH) is a derivative of hippuric acid and useful as a diagnostic agent for the measurement of renal plasma flow. About 20-30% is completely filtered by the glomerulus and not readsorbed by the tubules. The remainder which bypasses the glomerulus and enters the tubules is completely secreted. At low doses, PAH is almost completely removed with one-pass through the kidneys. Hence, the venous concentration of PAH is close to zero and PAH has been used to determine the effective renal plasma flow (eRPF) from the plasma. The venous concentration is usually <10% that of the plasma concentration so eRPF slightly underestimates the actual RPF. This error is generally accepted because of the ease with which PAH infusion allows calculation of eRPF:

$$eRPF = ([PAH]U/[PAH]P) * Urine\ Volume$$

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 -20ºC 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 section 5.

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)
TCA (15 %)	15 mL	-20°C	-20°C
DACA solution	15 mL	-20°C	-20°C
PAH Standard (10 mg/mL)	100 µL	-20°C	-20°C

Some of the products contained in this kit are corrosive and can cause severe damage if not handled properly. Please proceed with caution: use protective gear and perform experiment and preparations in a fume hood.

7. MATERIALS REQUIRED, NOT SUPPLIED

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

- MilliQ water or other type of double distilled water (ddH_2O)
- Microcentrifuge
- Pipettes and pipette tips
- Vortex
- Colorimetric microplate reader – equipped with filter for OD 550 nm
- 96 well plate: clear plates for colorimetric assay

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 **TCA (15%):**

Ready to use as supplied. Keep on ice while in use. Store at -20°C.

TCA is a corrosive product and should be handled with extra care and precaution. Please ensure you are wearing adequate protective gear and that you perform your work with this product in a ventilated fume hood.

9.2 **DACA solution:**

Ready to use as supplied. Keep on ice while in use. Store at -20°C.

9.3 **PAH Standard (10 mg/mL):**

Ready to use as supplied. Keep on ice while in use. 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 should not be stored for future use.

10.1 Prepare a 0.1 mg/mL PAH standard by diluting 5 µL of the provided PAH standard with 495 µL of ddH_2O.

10.2 Take 200 µL 0.1 mg/mL PAH standard and add 600 µL of the 15% TCA solution provided. Mix well.

10.3 Use the PAH standard/TCA mix to prepare the standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Standard (µL)	ddH_2O (µL)	Final volume standard in well (µL)	End Conc. PAH in well (µg/well)
1	0	150	50	0
2	30	120	50	0.25
3	60	90	50	0.50
4	90	60	50	0.75
5	120	30	50	1.00
6	150	0	50	1.25

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 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 your samples 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.
- Renal plasma flow determination requires both plasma and urine samples. For higher accuracy studies, we recommend plotting urine sample collection time and urine PAH concentration and compare it to plasma PAH concentration to be able to determine the plasma PAH concentration that best corresponds to the time the urine sample was obtained.

11.1 **Serum and Plasma samples:**

11.1.1 Dilute 50 µL EDTA plasma or serum with 50 µL of the TCA solution (15 %) provided in the kit.

11.1.2 Vortex and place on ice for 5 minutes.

11.1.3 Centrifuge samples for 2 – 5 minutes at 4°C at 10,000 x *g* using a cold microcentrifuge to remove any insoluble material.

11.1.4 For assay: use 50 µL of the clear supernatant per well.

11.2 **Urine samples:**

11.2.1 Dilute 50 µL with 50 µL of the TCA solution (15 %) provided in the kit.

11.2.2 Vortex and place on ice for 10 minutes.

11.2.3 Centrifuge samples for 2 – 5 minutes at 4°C at 10,000 x *g* using a cold microcentrifuge to remove any insoluble material.

11.2.4 For assay: take 10 µL and dilute with 190 µL distilled water. Use 50 µL/well for testing.

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.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

12.1 **Set up Reaction wells:**

- Standard wells = 50 µL standard dilutions.
- Sample wells = 50 µL samples (see Sample Preparation section for details on each sample type).

12.2 Add 150 µL of DACA into each standard and sample wells

12.3 Mix and incubate at room temperature for 30 min protected from light.

12.4 Measure output at OD 550nm on a microplate reader.

NOTE: *DACA can react with other aromatic amines and indoles. Common drugs such as sulfonamides and acetaminophen and their metabolites are among those which DACA can react with. If the presence of these is expected or suspected, the chemical should be tested for reaction with DACA at the concentrations expected.*

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 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.3 Plot the corrected absorbance values for each standard as a function of the final concentration of PAH.

13.4 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).

13.5 Concentration of PAH µg/mL in the test samples is calculated as:

$$PAH\ concentration = A * D$$

or

$$PAH\ concentration = \left(\frac{A}{V}\right) * 1000$$

If sample volumes other than recommended in this protocol are used

Where:

A = Amount of PAH determined from Standard Curve (in µg).

D = Sample dilution factor.

V = volume of sample added per well (in mL).

14. TYPICAL DATA

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

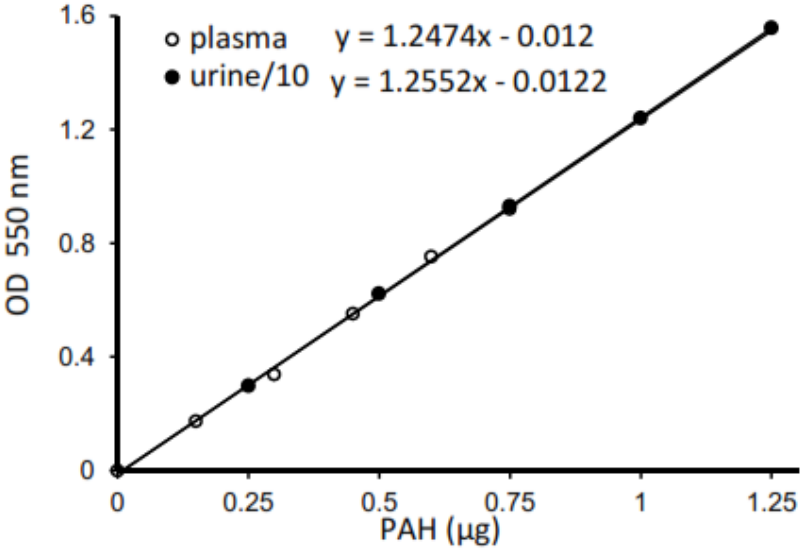


Figure 1. Typical PAH Standard calibration curve

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 standards (aliquot if necessary); get equipment ready.
- Prepare standard curve
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Set up plate for standard (50 µL) and samples (50 µL).
- Add DACA solution to wells (150 µL).
- Incubate plate at RT 30 minutes protected from light.
- Measure plate at OD 550 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 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

Problem	Cause	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) 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. FAQ

Do any chemicals or biological materials cause interference in this assay causing compromised results?

DACA can react with other aromatic amines and indoles. Common drugs such as sulfonamides and acetaminophen and their metabolites are among those which DACA can react with. If the presence of these is expected or suspected, the chemical should be tested for reaction with DACA at the concentrations expected.

18. NOTES

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