Carbamylation IP Kit

Item No. 601930

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Amount	Storage
601931	Carbamylation Affinity Sorbent (50% slurry)	1 vial/400 µl	4°C
601932	Anti-Carbamylation (Homocitrulline) Polyclonal Antibody	1 vial/150 µl	-20°C
601933	Carbamylated BSA	1 vial/100 µg	-20°C

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3640

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the Materials Supplied section (see page 3) and used before the expiration date indicate on the outside of the box. *Note: This kit ships at 4°C. Anti-Carbamylation (Homocitrulline) Polyclonal Antibody (Item No. 601932) and Carbamylated BSA (Item No. 601933) should be removed and stored at -20°C for best results.*

Materials Needed But Not Supplied

1. Microcentrifuge tubes (1.5 ml)
2. Cell lysis buffer with protease inhibitor
3. Wash buffer: PBS-T (PBS, pH 7.2, with 0.1% Tween)
4. Elution Buffer (as appropriate)
   - 1X SDS-PAGE Sample loading buffer for Western blot (WB) analysis
   - 0.1% Formic acid for proteomic analysis
5. Target protein-specific primary antibodies
6. Anti-rabbit AP- or HRP-conjugated secondary antibody (if using the Anti-Carbamylation (Homocitrulline) Polyclonal Antibody provided in the kit)
7. Reagents for WB

INTRODUCTION

Background

Carbamylation is a non-enzymatic and irreversible post-translational protein modification whereby cyanate/isocyanic acid adds a carbamoyl group to the ε-amino of lysine side chains, converting lysine to the non-standard amino acid homocitrulline.[1,2] Cyanate is formed primarily from the breakdown of urea but can also be formed from the oxidation of thiocyanate by myeloperoxidase in the presence of hydrogen peroxide at sites of inflammation, such as atherosclerotic tissues.[1-3] LDL can be carbamylated and has atherogenic properties, disrupting LDL receptor binding and increasing vascular smooth muscle cell proliferation and endothelial cell death.[3,4] Protein carbamylation and carbamylated LDL levels are increased in the plasma of patients with disorders resulting in high levels of urea, such as chronic and end-stage renal diseases.[2,4] Furthermore, the levels of plasma protein-bound homocitrulline positively correlate with the frequency of, and mortality from, major adverse cardiac events and predict cardiovascular disease risk.[3] Carbamylated proteins can also induce an immune response leading to the production of anti-homocitrulline autoantibodies, which have been found at higher levels in individuals with rheumatoid arthritis and are positively associated with the degree of joint damage.[5]

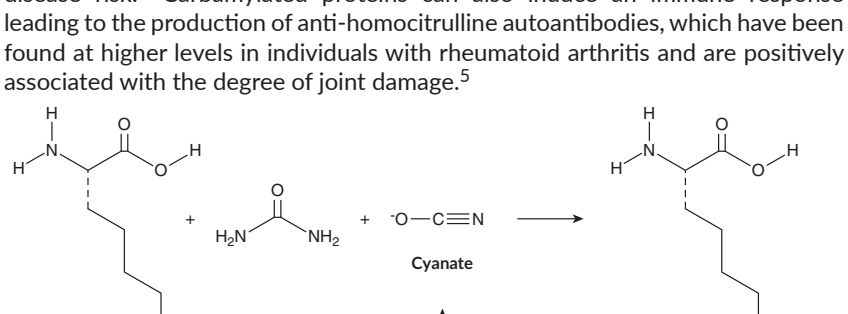

Figure 1. Carbamylation of lysine to homocitrulline

About This Assay

Cayman's Carbamylation Immunoprecipitation (IP) Kit provides a convenient method for the capture and concentration of carbamylated proteins from cell lysates using an anti-carbamylation monoclonal antibody coupled to agarose beads. Following elution of the captured proteins from the resin, they can be subjected to SDS-PAGE and analysis by WB using either the Anti-Carbamylation (Homocitrulline) Polyclonal Antibody included in the kit or a user-supplied target-specific antibody. A provided positive control sample included in the kit assures that the assay is performing appropriately.

1. Determine changes in lysine carbamylation in treated *versus* untreated samples.
2. Demonstrate target proteins are carbamylated *in vitro*.
3. Identify and characterize carbamylated proteins by WB and proteomic analysis.

PRE-ASSAY PREPARATION

Reagent Preparation

All reagents are ready to use as supplied unless otherwise specified.

Lysis Buffer

- Assay is compatible with a wide range of lysis buffers
- Avoid buffer components that cause protein denaturation
- Minimize use of reducing agents (*e.g.*, DTT) and detergents where possible
- Suggested lysis buffer: 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5% (v/v) NP-40, and protease inhibitor cocktail.

Elution Reagent

Select an appropriate elution reagent for intended method of analysis:

a) SDS-PAGE Sample loading buffer for WB analysis

b) 0.1% Formic acid for proteomic analysis

Sample Preparation

The recommended volume and concentration of sample/control lysate is 200 µl at 1-5 mg/ml as a starting point. Adjust lysate concentration with lysis buffer, if required. 500 ng of Carbamylated BSA is recommended as a positive control.

ASSAY PROTOCOL

Performing the Assay

Assay Optimization

Optimal assay conditions for the capture of carbamylated proteins from specific lysate samples must be determined by the user. Adjustment of the following parameters may facilitate this process:

- Sample volume: 100-500 µl
- Sample concentration: 1-5 mg/ml
- Carbamylation Affinity Sorbent volume: 40 µl suspension (20 µl settled resin)
- Assay time, minimum 1 hour to overnight

Keep reaction components on ice throughout set-up.

Carbamylation Affinity Sorbent Preparation

1. Re-suspend the Carbamylation Affinity Sorbent (Item No. 601931) by gentle inversion of the tube.
2. Aliquot 40 µl of the Carbamylation Affinity Sorbent suspension (20 µl settled resin) into the required number of microcentrifuge tubes.
3. Add 1 ml wash buffer to each tube.
   a. Mix with pipette.
   b. Centrifuge at low speed (500-1,000 x g, for two minutes) to collect matrix.
   c. Discard flow through.
4. Repeat matrix wash/collection at least twice.

Carbamylation Affinity Sorbent Assay

5. Add 200 µl of sample lysate or 500 ng Carbamylated BSA control to tubes.
6. Incubate for one hour at 4°C with rotary mixing.
7. Centrifuge at low speed (500-1,000 x g, for two minutes) to collect matrix.
8. Remove flow through and retain as 'Unbound Fraction' for subsequent analysis if required.
9. Replace column in collection tube.
10. Wash matrix by adding 1 ml wash buffer to tubes.
    - Centrifuge at low speed (500-1,000 x g, for two minutes) to collect matrix.
    - Repeat wash step three more times.

Elution of Captured Proteins

11. For SDS-PAGE/WB analysis:
    - Remove as much flow through as possible after the last wash without removing resin.
    - Add 20 µl of 1X SDS-PAGE sample buffer to the tube and mix by flicking with a finger.
    - Heat to 75°C for 10 minutes.
    - Centrifuge at low speed (500-1,000 x g, for two minutes) to collect eluted materials.
12. For proteomic analysis:
    - Remove as much flow through as possible after the last wash without removing resin.
    - Add 10 volumes (200 µl) of 0.1% formic acid to resin.
    - Rotary mix for 5-10 minutes at room temperature.
    - Centrifuge at low speed (500-1,000 x g, for two minutes) to collect eluted material.
    - Lyophilize the eluate and re-suspend in trypsin digestion or alternative buffer prior to subsequent processing analysis or store at -20°C.

ANALYSIS

Performance Characteristics

Western Blot Analysis

The Carbamylation IP Kit contains a rabbit polyclonal anti-carbamylation antibody for analysis by WB.

Variable	Recommendation
SDS-PAGE	10-20% gel
Sample loading	10 µl eluted reaction
Anti-Carbamylation (Homocitrulline) Polyclonal Antibody	1:200 dilution
Carbamylated BSA	500 ng
Secondary Antibody (user supplied)	Goat Anti-Rabbit (AP- or HRP Conjugate)
Target protein-specific antibody (user supplied)	WB conditions must be determined by the user and the antibody applied in conjunction with an appropriate secondary antibody

Example Assay Results

Lanes: *Protein amounts indicate the amount of protein loaded to the affinity sorbent*

Lane 1: BSA (0.5 µg)
Lane 2: Carbamylated BSA (0.5 µg)
Lane 3: HeLa Cell Lysate (10 µg)
Lane 4: Citrullinated HeLa Cell Lysate (10 µg)
Lane 5: Carbamylated HeLa Cell Lysate (10 µg)

Figure 2. Western blot analysis of the Carbamylation IP Kit. Carbamylated and control cell lysates, as well as the supplied positive control, were pulled down using the Carbamylation Affinity Sorbent, eluted, run on a 12% gel, transferred to nitrocellulose, and probed with the provided Anti-Carbamylation (Homocitrulline) Polyclonal Antibody. The data demonstrate that both the Affinity Sorbent and the Polyclonal Antibody recognize only the carbamylation proteins.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
No bands seen	Incorrect secondary antibody	Ensure appropriate secondary antibody is used for detection
No carbamylated proteins	Too little lysate	Add more lysate to matrix
Target protein not seen	Low abundance in lysate	Increase amount of sorbent

References

1. Stark, G.R., Stein, W.H., and Moore, S. Reactions of cyanate present in aqueous urea with amino acids and proteins. *J. Biol. Chem.* **235(11)**, 3177-3181 (1960).
2. Long, J., Vela Parada, X., and Kalim, S. Protein carbamylation in chronic kidney disease and dialysis. *Adv. Clin. Chem.* **87**, 37-67 (2018).
3. Wang, Z., Nicholls, S.J., Rodriquez, E.R., *et al.* Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nat. Med.* **13(1)**, 1176-1184 (2007).
4. Ok, E., Basnakian, A.G., Apostolov, E.O., *et al.* Carbamylated low-density lipoprotein induces death of endothelial cells: A link to atherosclerosis in patients with kidney disease. *Kidney Int.* **68(1)**, 173-178 (2005).
5. Shi, J., Knevel, R., Suwannalai, P., *et al.* Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. *Proc. Natl. Acad. Sci. USA* **108(42)**, 17372-17377 (2011).

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.