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ELISA Based Protein Ubiquitylation Measurement

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[Abstract] Ubiquitylation is a common post-translational modification of cellular proteins that results in proteasomal and lysosomal degradations. Ubiquitylation is generally measured by methods such as immunoblotting using anti-ubiquitin antibodies after isolating the protein-of-interest by denaturing immunoprecipitation. The following protocol can be used to easily quantify the ubiquitylation of the protein-of-interest tagged with biotin by ELISA.
Keywords: Ubiquitin, ELISA, Poly-ubiquitylation, Protein degradation, CFTR, Membrane protein

[Background] Protein ubiquitylation facilitates proteasomal and lysosomal degradations of misfolded proteins such as ΔF508 CFTR (Cystic Fibrosis Transmembrane conductance Regulator), resulting in the genetic disease, Cystic Fibrosis. In addition, protein ubiquitylation regulates selective autophagy, receptor endocytosis, and intracellular signal transductions. K48-linked poly-ubiquitylation and K63-linked poly-ubiquitylation facilitates proteasomal and lysosomal degradations, respectively. Therefore, quantitative analysis of protein ubiquitylation and specific poly-ubiquitin chain configurations of the protein-of-interest present as significant information in understanding the ubiquitylation-mediated processes.

Immunoblot detection of immunoprecipitated proteins is a commonly used technique to analyze protein ubiquitylation. This technique has an advantage in measuring the ubiquitylation of endogenous proteins; however, it is immensely demanding and time-consuming with relatively low sensitivity. In contrast, the ELISA-based ubiquitylation assay is simple and can easily quantitate the ubiquitylation levels of the protein-of-interest tagged with biotin when compared to immunoblot detection (Okiyoneda *et al.*, 2018). The protein biotinylation can be achieved by introducing an HB (histidine-biotin) tag (Tagwerker *et al.*, 2006) or AviTag™ on the protein-of-interest. The biotinylated protein can be easily immobilized on streptavidin or NeutrAvidin-coated 96 well plates and denatured by urea to dissociate the interacting proteins. We prefer NeutrAvidin-coated 96 well plates in this protocol due to NeutrAvidin which has a higher affinity and specificity against the biotin-tag compared to streptavidin. The ubiquitin (Ub) chains conjugated to the protein-of-interest can be quantitated by ELISA using anti-Ub antibodies, including poly-ubiquitin chain specific antibodies. Few ubiquitin ELISA kits are commercially available (*e.g.*, UbiQuantTM quantitative ubiquitin ELISA from LifeSensors) and few ubiquitin-related ELISA methods are published as well (Conte *et al.*, 2017). These published ELISAs use antibodies to immobilize ubiquitin or substrate proteins on multi-well plates. For some substrate proteins, the suitable antibody for the immobilization may be unavailable. In contrast, our method uses the interaction

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between Neutravidin and biotin in order to immobilize the biotinylated substrate proteins on multi-well plates. The interaction between Neutravidin and biotin exhibits the highest known affinity with high specificity. Moreover, it can withstand the presence of denaturing agents (*e.g.*, 2 M urea), which dissociates the substrate-binding proteins, which may also be ubiquitinated. Thus, our method is advantageous over the other methods due to the unnecessary use of antibodies for the immobilization and specific detection of the substrate ubiquitination. The ELISA-based ubiquitylation assay potentially detects ubiquitylation of endogenous proteins genetically introduced with an HB tag or AviTag™ by the CRISPR-Cas9 system. Through this protocol, we use the BHK cells stably expressing HBH (histidine-biotin-histidine) -ΔF508 CFTR-3HA which were generated as previous work (Okiyoneda *et al.*, 2018) while the ubiquitylation of ΔF508 CFTR tagged with an HBH tag can be measured by ELISA.

Materials and Reagents

1. 60 mm dish (Thermo Fisher Scientific, Thermo Scientific Nunc, catalog number: 150288)
2. 1.5 ml Hyper Microtube “High-Seal” (WATSON Bio Lab, catalog number: 131-815C)
3. Pierce NeutrAvidin coated 96-well white plate (Thermo Fisher Scientific, catalog number: 15116)
4. BHK-21 cells (ATCC® CCL-10™)
5. BHK cells expressing HBH (His-Biotin-His) and 3HA tagged ΔF508 CFTR (Okiyoneda *et al.*, 2018)
6. Antibodies

   1st antibodies

   a. Anti-Lys48 specific ubiquitin antibody (Apu2) (Merck KGaA, catalog number: 05-1307)
   b. Anti-Lys63 specific ubiquitin antibody (Apu3) (Merck KGaA, catalog number: 05-1308)
   c. Anti-HA tag antibody (16B12) (BioLegend, catalog number: 901515)

   2nd antibodies

   a. HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch, catalog number: 115-035-166)
   b. HRP-conjugated anti-rabbit IgG (Jackson ImmunoResearch, catalog number: 711-035-152)
7. Pepstatin A (Peptid institute, catalog number: 4397)
8. Leupeptin Hemisulfate monohydrate (FUJIFILM Wako Pure Chemical Corporation, catalog number: 122-03751)
9. PMSF (FUJIFILM Wako Pure Chemical Corporation, catalog number: 164-12181)
10. MG132 (Cayman Chemical, catalog number: 10012628)
11. N-Ethylmaleimide (Wako, catalog number: 054-02063)
12. NP-40 substitute (FUJIFILM Wako Pure Chemical Corporation, catalog number: 145-09701)
13. Urea (FUJIFILM Wako Pure Chemical Corporation, catalog number: 217-00615)
14. BSA (FUJIFILM Wako Pure Chemical Corporation, catalog number: 015-27053)

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15. SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher, catalog number: 34080)
16. Tris-HCl (FUJIFILM Wako Pure Chemical Corporation, catalog number: 204-07885)
17. NaCl (FUJIFILM Wako Pure Chemical Corporation, catalog number: 195-01663)
18. KCl (FUJIFILM Wako Pure Chemical Corporation, catalog number: 163-03545)
19. Na_2HPO_4 (FUJIFILM Wako Pure Chemical Corporation, catalog number: 197-02865)
20. KH_2PO_4 (FUJIFILM Wako Pure Chemical Corporation, catalog number: 169-04245)
21. SDS (FUJIFILM Wako Pure Chemical Corporation, catalog number: 191-07145)
22. Triton X-100 substitute (FUJIFILM Wako Pure Chemical Corporation, catalog number: 168-11805)
23. Sodium Deoxycholate (FUJIFILM Wako Pure Chemical Corporation, catalog number: 194-08311)
24. PBS (see Recipes)
25. RIPA buffer (see Recipes)
26. Lysis buffer (see Recipes)
27. Wash buffer (see Recipes)
28. Blocking buffer (see Recipes)
29. Denaturing buffer (see Recipes)
30. Urea wash buffer (see Recipes)

Equipment

1. Varioskan® Flash (Thermo Fisher Scientific, Thermo Scientific, catalog number: 5250040)
2. Wellwash™ Versa Microplate Washer (Thermo Fisher Scientific, Thermo Scientific, catalog number: 5165010)
3. 8 channel electronic pipette (Sartorius, catalog number: 730390) and 50-1,200 µl tips (Sartorius, catalog number: 791204)
4. Forma™ 310 Direct Heat CO_2 incubators (Thermo Fisher Scientific, Thermo Scientific, catalog number: 310, set at 37 °C with 5% CO_2)

Software

1. SkanIt™ (Thermo Fisher scientific)

Procedure

The scheme of the procedure is provided in Figure 1.

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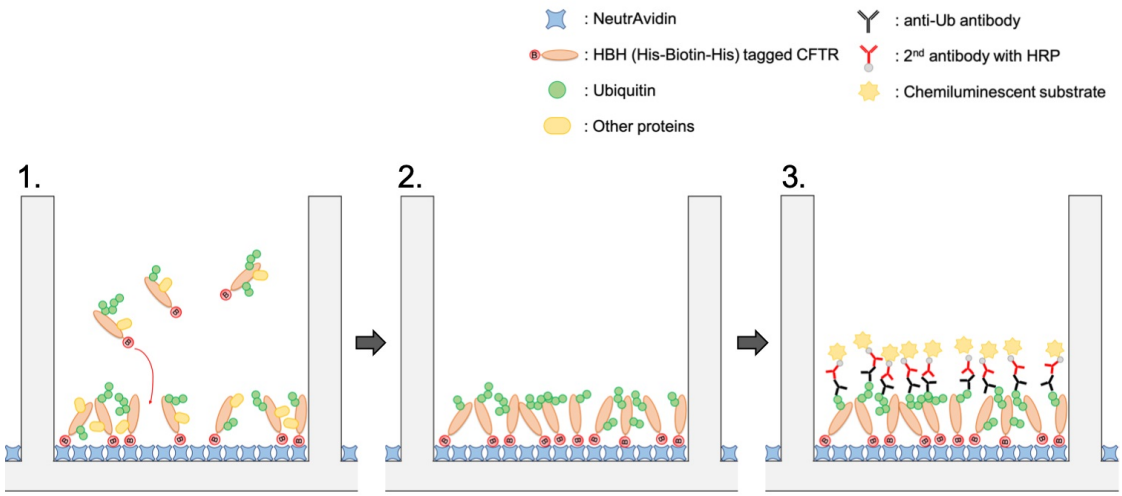

Figure 1. Scheme of the procedure of ELISA based protein ubiquitylation measurement. 1. Immobilize HBH tagged CFTR on NeutrAvidin coated 96-well plate. 2. The CFTR binding proteins (other proteins) are removed by adding the Denaturing buffer and washing with the Urea wash buffer. 3. Using an anti-Ub antibody (*e.g.*, anti-Lys48 specific ubiquitin antibody), the ubiquitylation is detected. Refer to the procedure for a detailed description.

A. Lysate preparation from BHK cells expressing Biotin-tagged CFTR
   1. Prepare confluent BHK cells (negative control) and BHK cells expressing Biotin-tagged target protein (*e.g.*, HBH-ΔF508-CFTR-3HA) on a 60 mm dish.
   2. Incubate with inhibitors (*e.g.*, 10 μM MG-132) for 3 h at 37 °C to accumulate the ubiquitylation.
   3. Lyse cells with 1 ml of lysis buffer (Recipe 3) on ice.
   4. Centrifuge (14,000 *x g*) for 15 min at 4 °C.
   5. Transfer 1 ml of supernatant to a fresh tube and place on ice for immediate assay or store at -80 °C for future usage.

B. Biotin-tagged CFTR immobilization on NeutrAvidin coated 96-well plate
   1. Wash the plate with the Wash buffer (400 μl/well) (Recipe 4).
   2. Block the plate with the Blocking buffer (100 μl/well) (Recipe 5) for 15-30 min on ice.
   3. Add the cell lysate to the plate according to Figure 2.

      Note: Add 50-150 μl/well of cell lysate depending on the desired signal for detection. It is essential to optimize the amount of cell lysate to obtain enough, but not saturated signals by the protein-of-interest. Prepare triplicate or quadruplicate samples for both CFTR and Ub detection. Negative control using cell lysate from BHK cells not expressing HBH-CFTR-3HA is necessary to measure the background signal.

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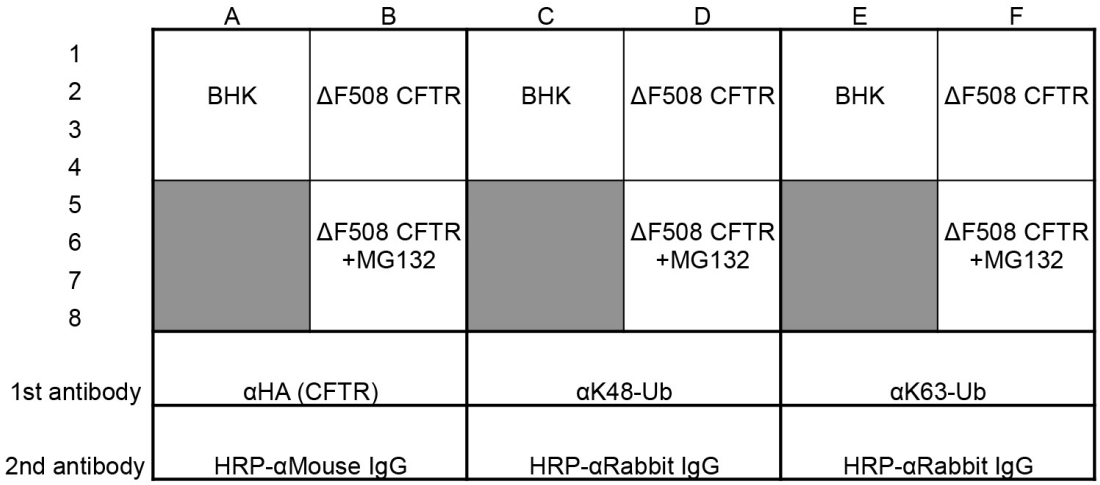

Figure 2. Assay template for ELISA based protein ubiquitylation measurement with negative control and two samples of HBH-ΔF508 CFTR-3HA in BHK cells. Cell lysate from BHK cells not expressing HBH-ΔF508 CFTR-3HA is used as a negative control to measure the background signal. BHK cells stably expressing HBH-ΔF508 CFTR-3HA were treated with 10 μM MG-132 for 3 h at 37 °C before the cell lysis.

4. Incubate for 2 h at 4 °C.
5. Wash the plate with the Wash buffer (400 μl/well) once.
   Note: If the target protein tends to aggregate, add detergent in all the steps.
6. Add Denaturing buffer (100 μl/well) (Recipe 6) and incubate for 5 min at RT (Room Temperature).
7. Wash the plate with the Urea wash buffer (400 μl/well) (Recipe 7) for 5 times.

C. Detection of the CFTR ubiquitylation by ELISA
1. Block the CFTR-immobilized plate with the Blocking buffer (100 μl/well) for 20 min at RT.
2. Add the primary antibody (50 μl/well) diluted with Blocking buffer according to Figure 2, and incubate for 1 h at RT.
   Note: For the primary antibody, the following conditions may work: Anti-Lys48 specific ubiquitin antibody (Apu2) (1:500); Anti-Lys63 specific ubiquitin antibody (Apu3) (1:500).
3. Wash the plate with the Wash buffer (400 μl/well) 4 times.
4. Add 50 μl/well of the secondary antibody diluted with the Blocking buffer according to Figure 2, and incubate for 45-60 min at RT.
   Note: For the secondary antibody, the following conditions may work: HRP-conjugated anti-mouse IgG (1:1000); HRP-conjugated anti-rabbit IgG (1:1000).
5. Wash the plate with the Wash buffer (400 μl/well) 6 times.
6. Add SuperSignal™ West Pico Chemiluminescent Substrate (100 μl/well) and incubate for 5 min at RT.
7. Read the luminescence signal by SkanIt™ software in Varioskan® Flash plate reader (Figure 3).

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8. The CFTR ubiquitylation level quantified by the anti-Ub antibody is normalized for the CFTR level quantified by the anti-HA antibody (Figure 4).

   Note: For normalization by the target protein, measure the amount of immobilized protein. If the target protein's signal is too strong, reduce the amount of cell lysate to prevent the saturation of the signal.

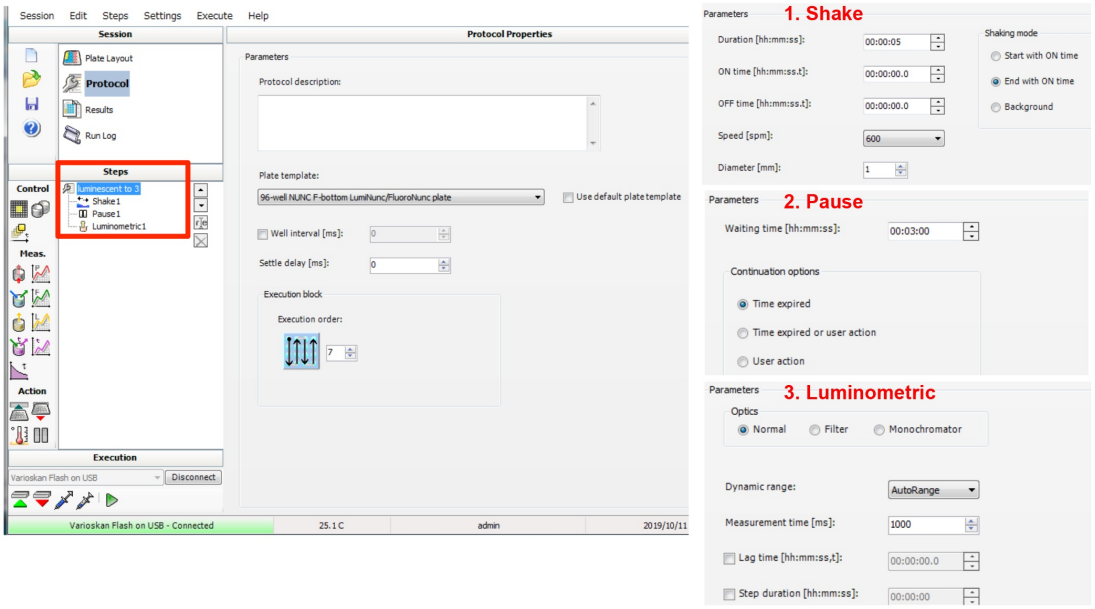

Figure 3. Protocol in SkanIt™ software. The luminescence signal is measured by the Varioskan® Flash plate reader with the SkanIt™ software. The protocol consists of 3 steps (boxed in red). 1st step is shaking the plate for 5 s. 2nd step is incubation (Pause) for 3 min. 3rd step is the luminescence measurement in a second.

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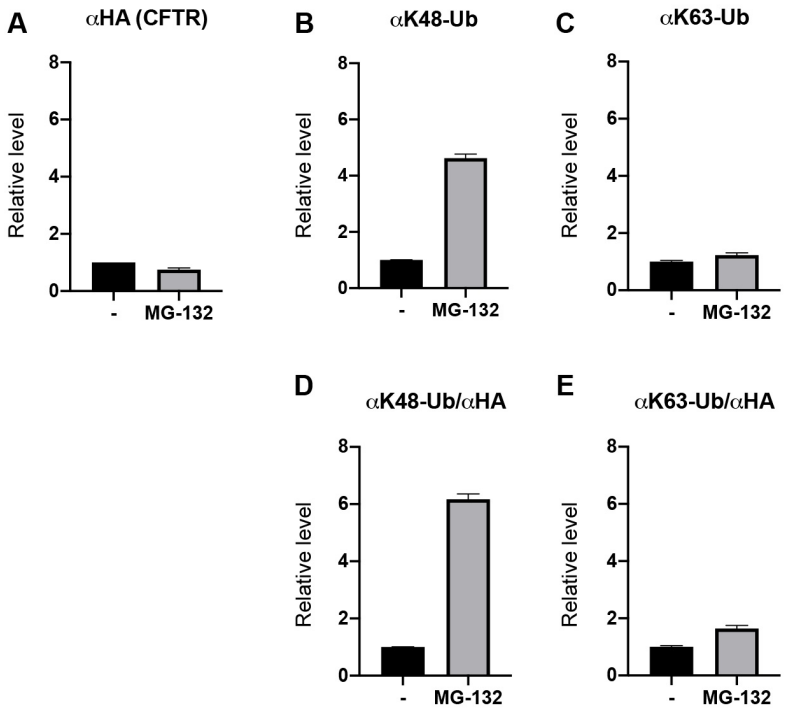

Figure 4. A typical result of the HBH-ΔF508 CFTR-3HA ubiquitylation in BHK cells by ELISA according to Figure 2. A. The amount of HBH-ΔF508 CFTR-3HA immobilized on the NeutrAvidin plate (8 μg/well cell lysate) was measured using anti-HA antibody. B-C. The amount of K48-linked poly-ubiquitin chains (B) (80 μg/well cell lysate) and K63-linked poly-ubiquitin chains (C) (80 μg/well cell lysate) on the immobilized CFTR was measured using anti-K48-Ub and anti-K63-Ub antibodies, respectively. D-E. The amount of K48-linked poly-ubiquitylation (D) and K63-linked poly-ubiquitylation (E) of HBH-ΔF508 CFTR-3HA was quantitated after the normalization for the CFTR level quantified by the anti-HA antibody.

Data analysis

1. Calculate the specific signal of the ubiquitin chains (*e.g.*, αK48-Ub) and CFTR (αHA) by subtracting the mean value of the background signal from the negative control.
2. Calculate the relative level of the specific signal (*e.g.*, αK48-Ub, αHA) of drug-treated cells (*e.g.*, MG-132) compared to the untreated control, as shown in Figures 4A-4C.
3. Calculate the relative level of ubiquitination by normalizing for the CFTR level (*e.g.*, αK48-Ub/αHA) as shown in Figures 4D and 4E.
4. For quantification, triplicate or quadruplicate experiments are repeated at least two times, while the data is expressed at means ± SEM. Statistical significance is assessed by two-tailed paired Student's *t*-test using Excel software (Microsoft).

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Recipes

1. PBS
   137 mM NaCl
   2.68 mM KCl
   8.1 mM Na_2HPO_4
   1.47 mM KH_2PO_4
2. RIPA buffer
   20 mM Tris-HCl (pH 8.0)
   150 mM NaCl
   0.1% SDS
   1% TritonX-100
   0.5% Sodium Deoxycholate
3. Lysis buffer
   RIPA buffer
   5 µg/ml Leupeptin
   5 µg/ml Pepstatin A
   100 µM PMSF
   20 µM MG132
   5 mM N-Ethylmaleimid
4. Wash buffer
   PBS
   0.1% NP-40
5. Blocking buffer
   PBS
   0.1% NP-40
   0.5% BSA
6. Denaturing buffer (freshly prepared)
   PBS
   8 M Urea
   0.1% NP-40
7. Urea wash buffer (freshly prepared)
   RIPA buffer
   2 M Urea

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Competing interests

The authors declare no competing financial interests.

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