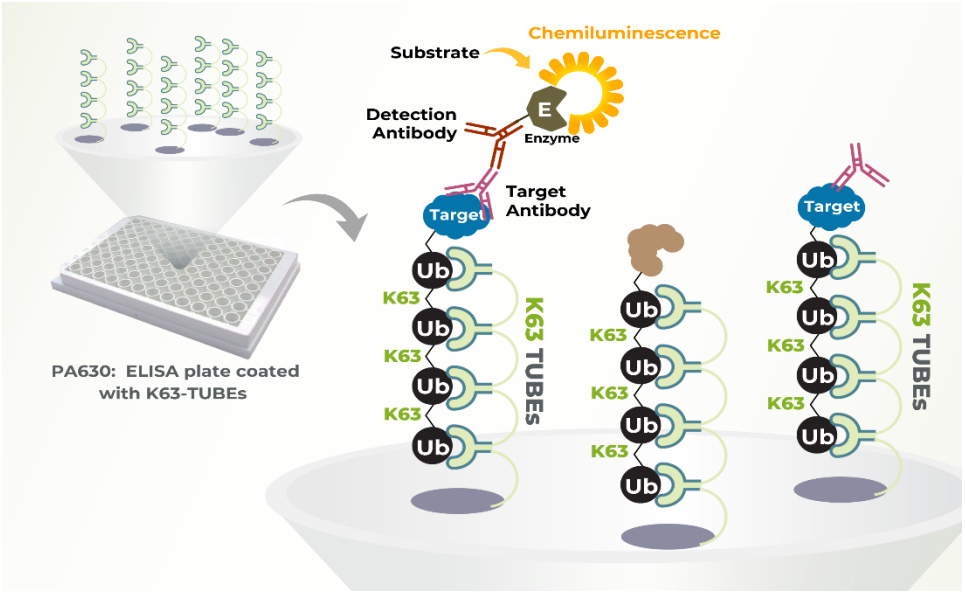
MANUAL

K63 Ubiquitin Linkage ELISA Kit (Chain Selective)
Catalog Number: PA630



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Cat. # PA630

BACKGROUND

The Ubiquitin Proteasome System (UPS) is a dynamic process involved in various cellular functions, including cell signaling, DNA repair, and proteasomal degradation. Ubiquitin can form diverse chains on its seven lysine residues, with K48 and K63-linked polyubiquitination being a crucial post-translational modification. While K48 polyubiquitination predominantly drives protein degradation and maintains cellular homeostasis, K63 linked polyubiquitination plays pivotal role in various cellular processes, primarily associated with trafficking, signaling pathways, DNA repair mechanisms and the regulation of protein-protein interactions.

IINTENDED USES

LifeSensors' K63 Ubiquitin Linkage ELISA kit is intended to enrich and quantitate K63 ubiquitination in cellular and tissue lysates or in in vitro ubiquitination reactions. This kit enables relative and absolute quantitation of K63 ubiquitination on target proteins in cellular and tissue lysates under various treatments (PROTACs, Molecular Glues, MG132, etc) and conditions. The plate is designed for research use only and is not intended for human or animal diagnostic or therapeutic applications.

BENEFITS

- Replaces labor-intensive, semi-quantitative Western blot methods for examining K63 polyubiquitination and protein degradation.
- Offers quantitative, reproducible results.
- Facilitates high-throughput screening for compound library processing and structure-activity relationship (SAR) studies.

PRINCIPLE OF THE ASSAY

Microtiter plate strips, pre-coated with a proprietary K63-TUBE reagent (assay plate) are used to capture K63-linked polyubiquitinated proteins in cell or tissue lysates or during an in vitro ubiquitination reaction. Non-polyubiquitinated proteins or those ubiquitinated on lysines residues other than K63 are either excluded from binding or removed by washing. The K63-ubiquitinated target Protein of Interest (POI) is detected with primary antibody against the POI and a secondary antibody conjugated with-HRP (Recommended R&D systems) allowing for detection by chemiluminescence (Figure 1). Thus, the signal generated in this "sandwich" ELISA assay is a quantitative measure of K63-linked ubiquitination of target proteins.

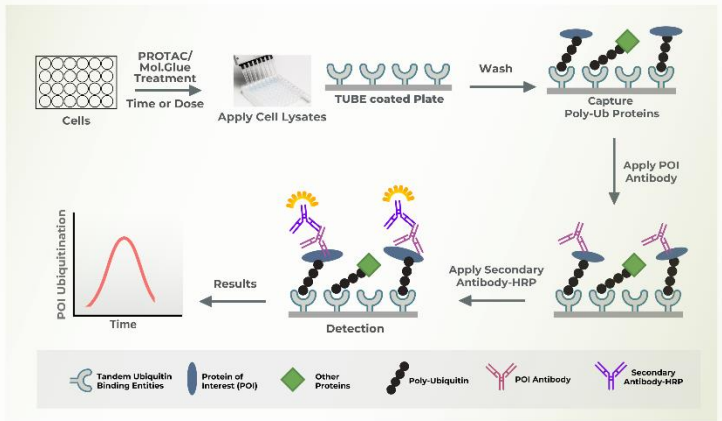

Figure 1. Schematic of K63 Ubiquitin Linkage ELISA Assay.

COMPONENTS INCLUDED IN THE KIT

1. ***K63-Ubiquitin Chain* Assay 96-well strip well plate**
   Note: Do not thaw the assay plate rapidly to room temperature. We recommend placing the assay plate at 4°C for 1 hour prior to transferring to room temperature.
2. **Blocking Concentrate (BC, 5X)** –1 x 12 mL
3. **Detection Reagent1 (DR1, 2X)** –1x 1.0 mL of Hydrogen Peroxide
4. **Detection Reagent 2 (DR2, 2X)**–1 x 1.0 mL of Luminol
5. **UPS Inhibitor cocktail (10X)** –1x 1.0 mL (For adding to the Lysis Buffer)
6. **Proteasome Inhibitor (10X**) –1x 1.0 mL (For adding to the Lysis Buffer)
7. **Plastic plate seals** –2 x

Positive Control Reagents:

8. **Biotin-K63-Poly-Ubiquitin Chain (20X):** –1 x 25 µL
9. **Streptavidin HRP (100X):** –1 x 10 µL

STORAGE AND STABILITY

Store Coated Plate at -80ºC. Store Blocking Concentrate and Detection Reagents at 4ºC. Store the UPS and Proteasome Inhibitor Cocktails at -20ºC. Plate seal can be stored at room temperature. Avoid multiple freeze/thaw cycle. All components are stable for at least 6 months if stored at the proper temperature.

ADDITIONAL ITEMS REQUIRED BUT NOT INCLUDED IN THE KIT

1. **Purified Target Protein of Interest if performing in vitro ubiquitination**
2. **PROTACs, Molecular Glues, MG132 etc for cell treatment or for in vitro ubiquitination reaction**
3. **ELISA-compatible anti-POI primary antibodies with matching HRP-conjugated secondary antibody.**
4. **WASH BUFFERS: 1X Phosphate Buffered Saline, 0.1% Tween (PBS-T)**
5. **Phosphatase inhibitors if studying phosphorylated target proteins of interest**
6. **LUMINESCENCE CAPABLE PLATE READER**
7. **15 mL centrifuge tubes**
8. **Polypropylene plate for preparing compound dilutions (Optional)**
9. **Multi-channel Pipettors and Automatic Plate Washer (Optional)**
10. **Disposable gloves, pipette tips and reagent reservoirs**

OPTIONAL

Decomplexing Buffer - SKU: UE-1003-2000. This buffer is available for purchase on the LifeSensors website here.

For some targets, the decomplexing agent enhances the signal to background. We recommend the use of the decomplexing agent prior to analysis with PA950, PA480 & PA630. The use of the decomplexing agent disrupts any native protein complexes that might be part of ubiquitin complexes. This urea based decomplexing agent results in reduced background signal, resulting in a better signal-to-background ratio.

DETAILED PROTOCOL

Note: It is essential that the entire manual is thoroughly reviewed before starting the procedure.

1. Remove the plate from freezer and reagents from the refrigerator, and place them at 4°C for at least 1 hour. Then allow them to reach room temperature (RT) for approximately 30 minutes. Incubations are performed at either 4°C or RT (22°-27°C). To avoid cross contamination, do not re-use plate sealers.
2. Prepare the cell lysate as described in the Cell Lysis Protocol. Decomplex the required amount (recommended 15 µg/well) using Decomplexing Buffer for 15 minutes, vortexing every 5 minutes. Then dilute the decomplexed lysate in PBS-T to a final volume of 100 µL per well. Add 100 µL of this diluted lysate to each well and seal the plate.
3. Incubate plate for 1.5 - 2 hours at RT with gentle orbital shaking (~300rpm).
4. Wash the assay plate 4 times with PBS-T using 200µL per well, tap out (upside down) excess PBS-T from wells between washes on paper towels or other blotting paper. NOTE: DO NOT ALLOW WELLS TO DRY COMPLETELY.
5. Prepare sufficient volume of 1X Blocking Concentrate by diluting 5X stock into 1X PBS-T.
6.
7. *Optional step:* To verify plate performance using the supplied positive control (Biotin-K63-polyubiquitin chain), prepare a solution of 1 ng/100 µL/well in 1X BC. Add this to at least 3 wells. Use 1X BC alone as the blank control for comparison.

    i. *Positive Control: Dilute the supplied Biotin-K63-polyubiquitin stock to 1 ng/100 µL using 1X BC Buffer. Prepare enough for at least three wells. Add 100 µL of the diluted positive control to three wells of the ELISA plate*
    ii. *Blank Control: In other three separate wells, add 100 µL of 1X BC Buffer alone to serve as blank controls. After incubation Detect using Streptavidin-HRP according to the assay protocol.*

8. Dilute the anti-POI primary antibody according to antibody manufacturer instructions in (1X) BC.

    i. *Note: Primary antibody dilution depends on primary antibody efficiency and manufacturer's recommendation. A good starting dilution is 1:1000 or 0.5-1 µg/mL.*

9. Pipette **100 µL** of diluted **anti-POI primary antibody** into each well.
10. *Optional step:* Pipette 100 µL of diluted Biotin-polyubiquitin chain (positive control) into three wells in assay plate. Pipette 100 µL 1X BC into three wells as blank control.
11. Seal the plate and incubate the assay plate for **60 minutes** at **room temperature** with shaking at 300RPM on an orbital shaker.
12. Remove the plate sealer and decant the reagents from the plate. Wash the assay plate 4 times with PBS-T using 200µL per well, tap out (upside down) excess PBS-T from wells between washes on paper towels or other blotting paper. NOTE: DO NOT ALLOW WELLS TO DRY COMPLETELY.
13. Dilute HRP-conjugated secondary antibody in 1x Blocking Concentrate and Dilute the Streptavidin-HRP to 1X in 1X BC for positive control detection.
14. Add the diluted secondary Antibody-HRP conjugate 100 µL/well. *Optional step:* Pipette 100µL of Streptavidin-HRP in 1X BC into wells containing positive control and blank. Incubate plate for 45 minutes at RT with gentle orbital shaking (~300rpm).

    i. *Note: Secondary Antibody-HRP conjugate should be diluted as recommended by manufacturer. A good starting dilution range is 1:1000 to 1:5000.*

15. Remove the plate sealer and decant the reagents from the plate. Wash the assay plate 4 times with PBS-T using 200µL per well, tap out excess PBS-T from wells between washes.
16. Just before use, mix 800 µL of DR1 and 800 µL of DR2 into 10 mL of ultrapure water (deionized or distilled). Add 100 µL of this detection reagent solution to each well and read immediately with a plate reader optimized for chemiluminescence for 5-10 reads with 1 min intervals.

    i. *Important Note: DR1 and DR2 amounts needed will vary based on overall signal and plate reader being used. Optimize the amount of DR1 and DR2 needed by reading with a 1:50 [DR1&DR2: ultrapure water] dilution of each reagent and then titrating up (1:20 to 1:10) based on initial raw values.*

CELL LYSIS PROTOCOL

1. For adherent cells aspirate medium completely and rinse cells with ice cold 1X PBS three times, add lysis buffer directly to the plate or wells and scrape cells into the lysis buffer (~75µl/well for a six-well plate). For suspension cells, spin down cells at ~1500 rpm for 3-5 minutes at 4°C. Gently aspirate media, wash the pellet with ice cold 1X PBS three times. Freeze cell pellet at -80°C for long term storage or proceed with lysis on ice.
2. For cell pellet, add RIPA lysis buffer (5-10 times the volume of the pellet, for example, for 100 µL of pellet add 500-1000 µL lysis buffer).
3. Vortex the cell lysate intermittently for 10-15 minutes while keeping the sample on ice to allow efficient lysis.
4. Centrifuge at 13,000 x g for 15-30 min at 4°C.
5. Collect the supernatant (whole cell lysate) and determine protein concentration using either Bradford or BCA method following manufacturer's recommendations.

RIPA Lysis Buffer

50 mM Tris-HCl, pH 7.5

150 mM NaCl

1% NP40

1% Sodium deoxycholate

2 mM EDTA

1 mM PMSF

10% Glycerol

1X UPS inhibitor cocktail (Add just before use)

1X Proteaome inhibitor cocktail (Add just before use)

1:500 Protease Inhibitor cocktail (Sigma Cat# P8849) (Add just before use)

NOTES

1. For Targeted protein degradation applications - to monitor K63 polyubiquitination of target protein followed by its degradation, please select times before and after D_{max} of PROTAC.
2. Optimize antibody dilution and specificity of target protein by western blotting. Selection of a good, clean target-specific antibody suitable for sandwich ELISA is essential for the success of this assay.
3. Optimize lysate concentration depending on target abundance.

4. See suggested lysis buffer below to have the best polyubiquitination profile of target protein.
5. Include appropriate controls for best results and interpretation.

REPRESENTATIVE DATA

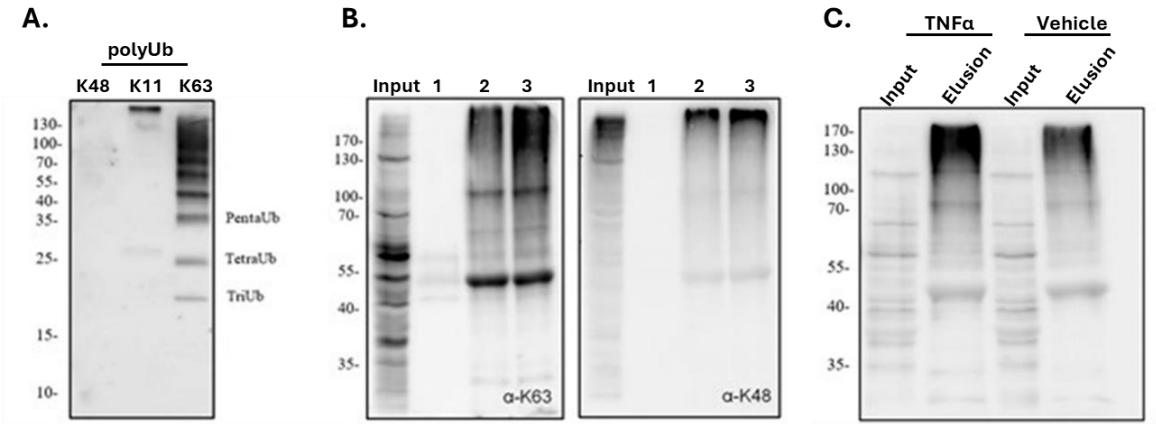

Figure 1: Validation of K63 ubiquitin linkage selective TUBEs (A) K48, K63 and K11 polyubiquitin chains were generated enzymatically followed by far western detection using K63 TUBE to investigate sensitivity and specificity of the TUBEs. Data represents good selectivity with K63 polyubiquitinated chains. (B) HEK293T cellular lysates used for IP applications using 0.5μM (2) or 1μM (3) FLAG® K63-TUBE. ~2mg total protein lysate was used to enrich K63 ubiquitinated proteome using anti-Flag M2 resin (10μl) for 3h (4°C). Enriched and eluted fractions are probed with commercial monoclonal antibodies specific for either K63- or K48-polyUb (Cell Signaling) to demonstrate K63 IP selectivity. Results indicate that K63 polyUb species are clearly enriched (α- K63), while the enrichment of K48 polyUb is relatively poor (α-K48). (C) Macrophages were isolated from murine bone marrow cultured for 8 days in DMEM supplemented with a combination of 10% FCS and conditioned media from the cultured murine L929 fibroblasts. Macrophages were then harvested and treated with either vehicle control or TNFα (20μg/ml) for 15 min to induce up regulation of K63-specific ubiquitylation. Enrichment of K63 ubiquitinated proteome using 500nM FLAG® K63-TUBE was performed analyzed by immunoblot using anti-K63 (α- K63).

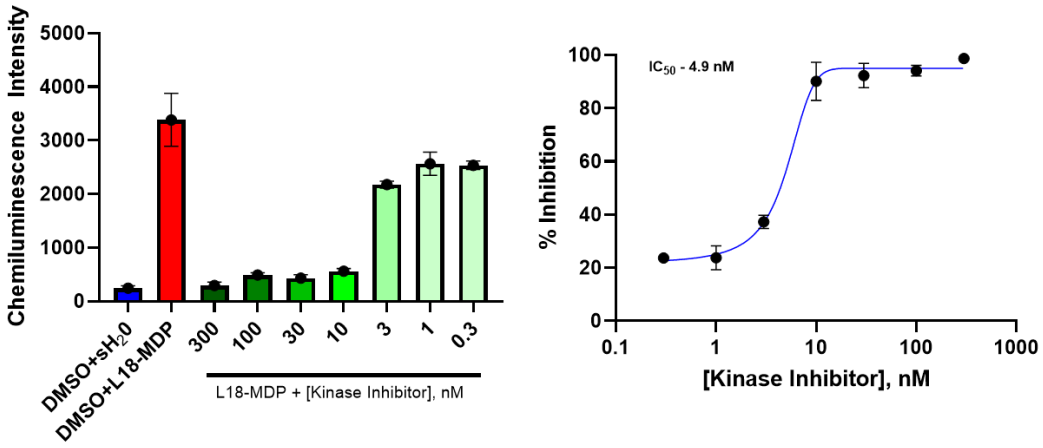

Figure 2: Validation of kinase inhibitor that inhibits K63 polyubiquitination on RIPK2 kinase using LifeSensors K63 Ubiquitin linkage ELISA Kit. RIPK2 K63 polyubiquitination triggered by 200ng/mg of L18-MDP (Lysine 18-muryldipeptide) treatment for 30 min in THP-1 cells. Analysis of levels of K63 polyubiquitination using PA630 ELISA kit. As expected, L18-MDP treatment resulted in enhanced levels of K63 ubiquitination on RIPK2 compared to vehicle control sterile water when probed with anti-RIPK2 on PA630 K63 TUBE plates. RIPK2 K63-polyubiquitination mediated by L18-MDP can be reversed in presence of kinase inhibitors. Linkage specific K63-TUBE ELISA kit clearly demonstrated effect of kinase inhibitors on RIPK2 inhibition reaching 100% inhibition at 100-300 nM of kinase inhibitors with IC50 values ~5nM consistent with literature. The data clearly suggests that PA630 can be used to selectively monitor levels of K63 polyubiquitination in presence of a stimulation like L18-MDP followed by monitoring inhibitory effects to predict IC50s crucial for reliable drug discovery.

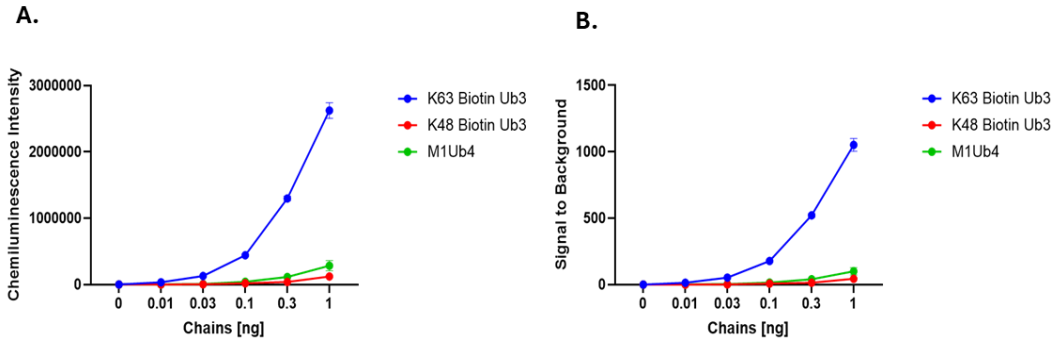

Figure 3: K63-Chain Selective ELISA Kit is highly specific for 63 ubiquitin linkage (A) Dose response of K63 and K48 biotinylated Tri-Ubiquitin chains and M1 biotinylated Tetra-Ubiquitin chains captured on ELISA Plates coated with K63 selective capture reagent detected using streptavidin HRP. (B) Signal to Background of K63 ubiquitin linkage ELISA assay with K63 and K4 biotinylated Tri-Ubiquitin and M1 biotinylated Tetra-Ubiquitin chains. Detection of ubiquitin chains was performed with streptavidin HRP.

TROUBLESHOOTING GUIDE

Below are common problems, possible causes, and recommended solutions to help you achieve reliable and reproducible results.

1. Weak or No Chemiluminscence Signal

Possible Causes & Solutions:

- Poor primary antibody performance → This can be a major hurdle in getting optimal results. Verify that the 1° antibody is validated for ELISA and titrate as needed. A poor antibody can lead to non-specific signals. Try different lot of antibodies.
- Dry Plate - Do not allow the plates to completely dry during any step of the procedure. Immediately add new solutions after all washes.
- Detection Reagent concentration too low → Titrate detection reagent to higher concentration in order to increase saturation and signal.

2. High Background Signal or Non-Specific Ubiquitination

Possible Causes & Solutions:

- Insufficient washing between steps → Inadequate washing can leave non-specific proteins on the plate, leading to high background. Ensure proper plate washing using the recommended buffer and multiple wash steps.
- Excess Cell Lysate → Optimize lysate concentrations to obtain best signal to background
- Primary antibody concentration too high → Titrate the antibody to reduce non-specific binding.
- Non-specific ubiquitination of other proteins → Run a negative control (no cell lysate) to confirm specificity.
- Contaminated reagents or buffers → Use fresh reagents and ensure proper storage.
- If using a plate washer to wash the plates, use freshly made wash buffer.
- Bubble Formation - Avoid creating bubbles. Remove bubble before taking the readings as bubbles can interfere with the readings.
- Detection Reagent Concentration too high → Titrate detection reagent down in order to lessen the saturation allowing for a bigger difference between samples

3. Unexpected Ubiquitination Patterns

Possible Causes & Solutions:

- Cell lysate instability → Avoid multiple freeze-thaws to prevent unnecessary proteolysis.

4. Inconsistent Results Between Experiments

Possible Causes & Solutions:

- Variation in cell lysate or reagent concentrations → Always prepare master mixes and use consistent cell lysate samples.
- Temperature fluctuations → Maintain a stable incubation temperature throughout the assay.
- Improper washing of plates → Inconsistent washing can cause variability in signal intensity. Ensure uniform, thorough washing between steps.
  Poor pipetting accuracy → Use calibrated pipettes and low-retention tips to ensure precise reagent additions. Use a multichannel pipette for quick and uniform addition of solution to wells.
- Cross-contamination between wells → Be cautious when adding reagents to prevent splashing or cross-contamination between wells.
- Incomplete Reactions → Ensure that the reagents and samples are added to the bottom of the well. Complete mixing of solutions in the well is critical.

5. Poor Reproducibility Across Experiments or Between Users

Possible Causes & Solutions:

- Inconsistent incubation times → Use a timer to ensure precise reaction timing between experiments.
- Variation in reagent lot numbers → If switching reagent lots, validate the new batch by running side-by-side comparisons.
- Improper mixing of reagents → Always mix reagents gently but thoroughly to avoid gradients in concentration within the wells.
- User variability in pipetting technique → Train all users on consistent pipetting methods and consider using multi-channel pipettes to minimize variability.

Final Tips for Experimental Success

- Run Controls – Always include positive and negative controls to validate results.
- ***Use High-Quality Antibodies*** – Poor antibodies can produce misleading data. Choose ELISA-validated antibodies with strong supporting data.
- Optimize Titrations – Proper titration of cell lysate, primary antibody, and detection reagent is essential for high-quality results.
- Check Reagent Stability – Ensure cell lysate samples are stored correctly to prevent degradation.
- Repeat Key Experiments – If results are inconsistent, repeat experiments with fresh reagents and carefully controlled conditions.
- Wash the Plate Properly – Inadequate washing between steps can lead to high background signals and retention of non-specific proteins. Be sure to follow recommended washing steps, including multiple wash cycles, to ensure cleaner results.

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2. use of the product or its derivatives to provide a service, information, or data,
3. use of the product or its derivatives for diagnostic purposes,
4. transfer or sale of protein or chemical component made with the product or derivatives of the product,
5. re-sale of the product or its derivatives, whether or not such product or its derivatives are resold for use in research.

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