Flag-tag Protein IP Assay Kit (Agarose Gel)

Introduction

The Flag tag is a polypeptide consisting of 8 amino acid residues (DYKDDDDK), usually in the form of Flag or 3X Flag. It can be linked to the 5' or 3' end of the gene of interest through genetic recombination technology to form a target protein with a Flag tag. Flag tags are widely used in the study of protein expression, purification, identification, interaction and function because they do not interact with the target protein and do not affect the function of the target protein, the N-terminal Flag tag can be excised by enterokinase to obtain the untagged target protein, and can be detected and purified by Flag antibody, Anti-Flag gel or Anti-Flag affinity gel.

This kit contains high-quality Anti-DYKDDDDK immunogel and optimized and validated necessary reagents for immunoprecipitation, making immunoprecipitation (IP, also known as pull-down) or co-immunoprecipitation (Co-IP) experiments easier and more efficient, and widely used in immunoprecipitation, co-immunoprecipitation or purification experiments of Flag-tagged fusion proteins or their protein complexes. It can be applied to a variety of sample types, such as cell lysates, cell secretion supernatants, serum, animal ascites, and other immune antigens.

This product can be used for immunoprecipitation (IP) and co-immunoprecipitation (Co-IP). Available in 10 tests and 50 tests (based on a single dose of 20 μL of Anti-DYKDDDDK immunogel).

Components and Storage

Components \ Size	10 tests	50 tests	Storage
Anti-DYKDDDDK (Flag) Agarose Gel	200 μL	1 mL	4°C
Cell Lysis Buffer	5 mL	25 mL	4°C
10X TBS	5 mL	30 mL	4°C
Protease Inhibitor Cocktail (100X)	50 μL	250 μL	-20°C
5X Protein Loading Buffer (Reducing)	200 μL	1 mL	-20°C
Acid Elution Buffer	1 mL	5 mL	4°C
Neutralization Buffer	100 μL	500 μL	4°C
3X Flag Peptide（25X）	80 μL	0.4 mL	-20°C
Shipping: Blue ice	Shelf life: 12 months		

Protocol

1. Preparation of experimental reagents

Prepare the amount of relevant reagents for this experiment according to the table below - prepare the relevant reagents at a ratio of 100/500 μL of lysate per sample

steps	Reagent preparation	Volume 1	Volume 2
Sample and cell lysis	Cell lysis (with Cocktail)	100 μL	500 μL
Preparation of 3X Flag elution buffer and gel	1X TBS	~0.52 mL	~1.6 mL
Immunoprecipitation, IP	Anti-Flag Agarose Gel	4 μL	20 μL
Cleaning (3 times)	Cell lysis (with Cocktail)	100 μL/time	500 μL/time
Competitive elution of tag peptides (optional)	3X Flag Tag Peptide Eluent (1X)	20 μL	100 μL
Acid elution and neutralization (optional).	Acidic eluent	20 μL	100 μL
	Neutralizing solution	2 μL	10 μL
SDS-PAGE protein elution with Loading Buffer (optional)	Protein Loading Buffer (1X)	20 μL	100 μL

The configuration is as follows:

a. Cell lysis (with Cocktail)：Cell Lysis Buffer (K1124) was mixed with Protease Inhibitor Cocktail (100X) in a 100:1 ratio, e.g., 10 μL of Protease Inhibitor Cocktail (100X) was added to 1 mL of Lysis Buffer to yield 1 mL of inhibitor-containing lysate. The prepared lysate should be placed in an ice bath or 4°C for later use.

***Note:**

1: If the target protein of immunoprecipitation involves phosphorylation modification or acetylation modification, phosphatase inhibitor or deacetylase inhibitor needs to be added. Phosphatase inhibitor cocktail A (K1015/K4004) and deacetylase inhibitor cocktail (K1017) are recommended. If there is a special need, consider choosing an appropriate inhibitor cocktail.

2: The Cell Lysis Buffer provided with this kit is not only used for sample lysis, but also for subsequent washing steps.

3: The lysate (including inhibitor Cocktail) is recommended to be prepared now, and it is not suitable to be frozen and stored for subsequent use after preparation.

b. 1X TBS: Dilute 10X TBS to 1X with ultrapure water. For example, 1 mL of 10X TBS is mixed with 9 mL of ultrapure water.

c. Anti-Flag Agarose Gel: Follow Agarose Gel Pretreatment (Step 4)

d. 3X Flag Tag Peptide Eluent (1X): 3X Flag Peptide Eluent is diluted 25-fold with 1X TBS, e.g., 10 μL of 3X Flag Peptide (25X) is mixed in 240 μL of TBS. The prepared 3X Flag peptide eluent should be placed in an ice bath or 4°C for later use. 3X Flag Peptide (25X) is optimized for most elutions and can be diluted only 10-fold if the tag protein is abundant. For more 3X Flag peptides, we recommend purchasing 3X Flag Peptide (A6001).

e. Acidic eluent and Neutralizing solution: Acid Elution Buffer and Neutralization Buffer.

f. Protein Loading Buffer (1X)：Take an appropriate amount of 5X Protein Loading Buffer (Reducing) (K1164)

and dilute it 5 times with water to obtain Protein Loading Buffer (Reducing) (1X). For example, 0.2 mL of 5X Protein Loading Buffer (Reducing) is mixed with 0.8 mL of ultrapure water.

2. Preparation of samples

- Adherent cells: Aspirate the culture medium. If necessary, wash once with PBS (Cat. #: H2018) and then aspirate the residual liquid. Add 100-200 μL of lysate (containing inhibitor cocktail) per 50-1 million cells (equivalent to one well of a 6-well plate) and pipette appropriately to allow the lysate to fully contact the cells. Cells are usually lysed 1-2 s after the lysate comes into contact with animal cells. Plant cells should be lysed on ice for 2-10 min. After full lysis, 10,000-14,000×g was centrifuged at 4°C for 3-5 min, and the supernatant was taken for subsequent immunoprecipitation and co-immunoprecipitation. The supernatant collected by centrifugation can be kept on ice for later use or stored at -20 °C for long-term storage.

***Note:** A small amount of insoluble material, mainly genomic DNA, will appear after lysis, and precipitate will be produced after centrifugation.

- Cells in suspension: Collect cells by centrifugation at 250-1,000 ×g for 3-5 minutes at room temperature. If necessary, PBS (Cat. #: H2018) can be used to wash once, then aspirate the residual liquid and gently vortex or flick the bottom of the tube to spread the cells as far apart as possible. Add 100-200 μL of lysate (containing inhibitor cocktail) per 50-1 million cells (equivalent to one well of a 6-well plate). Flick the bottom of the tube or pipette appropriately to fully lyse the cells. After full lysis, centrifuge at 10,000-14,000×g at 4°C for 3-5 min, take the supernatant, and then proceed to subsequent immunoprecipitation and co-immunoprecipitation. The supernatant collected by centrifugation can be kept on ice for later use or stored at -20 °C for long-term storage.

***Note:**

1: There should be no significant cell pellet after adequate lysis.

2: If the number of cells is large, it is recommended to aliquot into 50-1 million cells/tube, and then lyse; Large clumps of cells are more difficult to fully lyse, while a small number of cells are relatively easy to fully lyse due to the easy contact between the lysate and the cells.

3: After lysis, a small amount of insoluble substances will appear, mainly genomic DNA, etc., and precipitates will be produced after centrifugation.

- Bacterial or yeast samples: For 1 mL of bacterial or yeast solution, centrifuge to remove the supernatant and, if necessary, use PBS (Cat. #: H2018Wash once and then blot off any remaining liquid. Gently vortex or flick the bottom of the tube to disperse the bacteria or yeast as much as possible. Add 100-200 μL of lysate (containing inhibitor Cocktail), gently vortex or bounce the bottom of the tube to mix, and lyse on ice for 2-10 minutes. For better lysis, bacteria and yeast can use lysozyme and wall-breaking enzyme (Lyticase), respectively) and then lysed using inhibitor-containing lysate. After full lysis, 10,000-14,000×g was centrifuged at 4°C for 3-5 min, and the supernatant was taken for subsequent immunoprecipitation and co-immunoprecipitation. Note: A small amount of insoluble material, mainly genomic DNA, is likely to appear after lysis, and a precipitate will be produced after centrifugation. The supernatant collected by

centrifugation can be kept on ice for later use or stored at -20 °C for long-term storage.

***Note:** A small amount of insoluble material, mainly genomic DNA, will appear after lysis, and precipitate will be produced after centrifugation.

- Tissue samples: Large pieces of tissue are first sheared into fine pieces and lysate (with inhibitor Cocktail) is added at a ratio of 100-200 μL per 10-20 mg of tissue. Homogenize with a glass homogenizer or grind with a tissue grinder until fully lysed, or freeze the tissue sample and grind it with liquid nitrogen, and add lysis and washing solution for lysis after sufficient grinding. After full lysis, 10,000-14,000×g was centrifuged at 4°C for 3-5 min, and the supernatant was taken for subsequent immunoprecipitation and co-immunoprecipitation. The supernatant collected by centrifugation can be kept on ice for later use or stored at -20 °C for long-term storage.

***Note:**

1: If the lysis is not sufficient, more inhibitor-containing lysates can be used, and if a high concentration of protein samples is required, the amount of lysate can be appropriately reduced.

2: The protein concentration of the supernatant obtained after lysis with 200 μL of lysate (containing inhibitor Cocktail) per 20 mg cryopreserved mouse liver tissue varies from tissue to tissue in different states.

3: After lysis, a small amount of insoluble substances will appear, mainly genomic DNA, etc., and precipitates will be produced after centrifugation.

3. Pretreatment of Anti-DYKDDDDK (Flag) Agarose Gel

Since Anti-DYKDDDDK (Flag) Agarose Gel is stored in a special protective solution, it needs to be properly washed before adding to the sample.

a. Resuspend the Anti-DYKDDDDK (Flag) Agarose Gel by gently pipetting and dispense 20 μL of the magnetic bead suspension per 500 μL sample into a clean 1.5 mL centrifuge tube.
b. Add 1X TBS to a final volume of 500 μL, cap the tube, mix by inverting several times or vortexing lightly for 1 minute, and centrifuge at 1,000 rpm for 5 minutes to remove the supernatant.
c. Repeat step (3.b) 2 times above.

4. Immunoprecipitation, IP

a. Add 500 μL of the sample prepared in step 2 to the pretreated gel and incubate on an inverted mixer (2 h at room temperature or 4°C overnight);
b. The mixture was centrifuged at 1,000 rpm for 5 minutes, and the supernatant was transferred to a new tube for later use (the supernatant could be used to detect the presence of carryover of the DYKDDDDK-tagged protein), leaving the protein-gel complex in the original tube.

5. Wash

a. In step (4.b), 500 μL of lysate (containing inhibitor Cocktail) was added to the compound species, gently pipetted and resuspended, and then placed on a magnetic stand for 1 minute to remove the supernatant.

b. Repeat the previous step 3 times until the OD280 of the washed supernatant is less than 0.05.

***Note:** If the supernatant OD280 is greater than 0.05, the number of washes should be increased appropriately.

6. Elution

According to the characteristics of the tagged protein and the requirements of subsequent experiments, one of the following three methods can be selected for elution.

- **3X Flag competitive elution method**: This method is a non-denaturing method, with high elution efficiency, and the eluted protein retains the original biological activity, which is convenient for subsequent analysis and detection.
  a. Add 100 μL of 3X Flag-tagged peptide eluent (1X) per 20 μL of original gel volume, mix well, place on a side swinger or rotary mixer, and incubate for 30–60 minutes at room temperature with shaking, or 1–2 h at 4°C . To improve elution efficiency, the incubation time can be extended or the elution can be repeated.
  b. After incubation, centrifuge at 1000 rpm for 5 minutes to separate the gel and collect the supernatant into a new centrifuge tube. The supernatant is the eluted Flag-tagged protein.
  c. The eluted Flag-tagged protein is stored at 4°C or -20°C or -80°C for long-term storage.
- **Acid elution method**: This method is a non-denaturing method, which is relatively fast and efficient. The eluted protein retains its original biological activity, which is convenient for subsequent functional analysis and detection.
  a. Add 50–100 μL of acidic eluent to the washed protein-gel complex species and incubate at room temperature for 5–10 minutes, but not more than 15 minutes.
  b. After incubation, centrifuge at 1000 rpm for 5 minutes to separate the gel and collect the supernatant into a new centrifuge tube.
  c. 10 μL of neutralization solution was added per 100 μL of eluent, and the pH of the eluate was adjusted to neutral for later functional analysis.
  d. Elute and neutralize Flag-tagged proteins and store at 4°C for later use, or at -20°C or -80°C for long-term storage.

***Note:**

1: Acid elution, while efficient, may still be lower than competitive elution or SDS-PAGE loading buffer elution.

2: Because the difference of the target protein may have a certain impact on the elution efficiency of the acid elution method, if the requirements for the elution efficiency are relatively high, the pH of the acid eluent can be adjusted between 2.5-3.1, and the pH value or amount of the corresponding neutralization solution should also be adjusted to a certain extent.

- **SDS-PAGE loading buffer elution method**: This method is a denaturing method, and the obtained protein samples are suitable for SDS-PAGE electrophoresis or WB detection.
  a. For every 20 μL of raw gel volume, 100 μL of Protein Loading Buffer (1X) was added and heated at 100°C for 10 min. The loading buffer contains a reducing agent such as DTT, and the eluted protein

sample will contain the light and heavy chains of the Flag antibody.

b. After cooling, the gels were separated by centrifugation at 1000 rpm for 5 minutes, and the supernatants were collected into new centrifuge tubes, followed by SDS-PAGE electrophoresis or Western blot.

FAQs

1. Resolve the gel adhesion to the tube wall

   A: Use low-adsorption consumables: When performing gel manipulations, it is recommended to use experimental consumables with low adsorption rates.

2. Antigen not precipitated problem

   A:1. Insufficient antigen content: Ensure that the antigen content in the sample is sufficient, which can be verified by SDS-PAGE or Western blot; 2. Increase the amount of sample: if necessary, increase the amount of sample; 3. Buffer composition interference: Try using other buffers for immunoprecipitation and rinsing.

3. The amount of protein is too low

   A:1. Protein degradation: protease inhibitors are added to prevent protein degradation; 2. Increase the amount of gel: If the amount of Anti-DYKDDDDK immunogel used is insufficient, consider increasing the dosage.

4. Non-specific banding issues

   A: Non-specific protein binding: Add 50~350 mM NaCl to the binding/washing buffer and increase the number and intensity of elution.

Precautions

1. This product should be maintained at pH 6-8 and avoid high-speed centrifugation and drying.
2. Before using this product, it should be properly and fully resuspended, that is, inverted several times to mix the gel evenly, and the mixing operation should be gentle, and it should not be violently vortex and shake to avoid antibody denaturation.
3. At the time of immunoprecipitation or purification, it is recommended to set up positive and negative control groups.
4. Purification of protein samples should be completed as soon as possible after collection and should always be placed at 4°C or in an ice bath to slow down protein degradation or denaturation.
5. If you use an instrument such as a vacuum pump to aspirate the supernatant, you must pay attention to the

suction strength of the vacuum pump to avoid excessive suction and sucking up the aggregated gel.

6. When the acidic solution is eluted, the gel may aggregate, which is a normal phenomenon and does not affect the normal use of the gel. A nonionic detergent of 0.1% (e.g., Triton X-100, Tween-20, or NP-40) effectively prevents gel aggregation and does not affect the antibody binding efficiency of the gel.

7. This product is for scientific research use only.

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