Technical Bulletin

FLAG® Immunoprecipitation Kit

FLAGIPT1

Product Description

Immunoprecipitation (IP) is a widely performed procedure for the isolation of proteins or protein complexes.[1-3] IP consists of multiple ordered steps:

- Cell lysis
- Binding of specific antigen to an antibody
- Antibody-antigen complex precipitation
- Precipitant wash
- Antigen dissociation from the immune complex

Epitope-tagged proteins can be affinity-purified and immunoprecipitated, using highly specific antibodies raised against their epitope. The use of such antibodies facilitates subsequent biochemical and immunological analysis.

The FLAG® epitope system relies on the FLAG octapeptide (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C; DYKDDDDK), which allows fusion proteins to retain their original conformation and function. The hydrophilic character of FLAG® increases the likelihood that it will be located on the surface of the fusion protein where it is accessible to antibodies.

The FLAG® Immunoprecipitation Kit allows rapid and efficient immunoprecipitation and elution of an active FLAG® fusion protein. The immunoprecipitation is performed with ANTI-FLAG®-M2 affinity gel, which is a highly specific monoclonal antibody covalently attached to agarose resin. The use of affinity resin allows efficient binding of FLAG® fusion proteins without the need for preliminary steps and calibrations. The immunoprecipitated FLAG® fusion proteins can be efficiently eluted from the resin with acidic conditions or by competition with the FLAG® peptide.[4-6] The immunoprecipitated proteins can be detected for their size, post-translational modifications and interactions on gel electrophoresis, and by activity assays.

Several publications cite use of the FLAGIPT1 kit in their protocols.[7-15]

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Reagents

Sufficient for 50 immunoprecipitation reactions

- Lysis Buffer [Component Number L3412; 50 mM Tris HCl (pH 7.4), with 150 mM NaCl, 1 mM EDTA, and 1% TRITON® X-100]: 50 mL
- 10× Wash Buffer [Component Number W0390; 0.5 M Tris HCl (pH 7.4) with 1.5 M NaCl]: 30 mL
- Elution Buffer [Component Number E6150; 0.1 M Glycine (pH 3.5)]: 30 mL
- 2× Sample Buffer [Component Number S8684; 125 mM Tris HCl, pH 6.8, with 4% SDS, 20% (v/v) glycerol, and 0.004% bromphenol blue]: 1.5 mL
- 3X FLAG® Peptide (Cat. No. SAE0194): 1 mg
- ANTI-FLAG® M2-Agarose Affinity Gel (Cat. No. A2220): 1 mL
- Amino-terminal FLAG-BAP™ Fusion Protein (Component Number I7582): 50 µg

Reagents and Equipment Required but not provided

(Cat. Nos. are given where appropriate)

- Test tubes
- Hamilton® syringe (such as Cat. No. 24539) or a Pasteur pipette (such as Cat. No. S6268)
- Shaker
- Microcentrifuge
- Dulbecco's Phosphate Buffered Saline (PBS), such as Cat. No. D8537
- Cell scrapers, such as Cat. No. CLS3010
- Protease Inhibitor Cocktail, such as Cat. No. P8340
- SIGMA*FAST*™ pNPP substrate tablets set (Cat. No. N1891), or pNPP liquid substrate system (Cat. No. N7653)

Preparation Instructions

Preparation of working solutions

- Thaw each kit component. Ensure homogeneity of each component, such as with gentle swirling.
- Before use, equilibrate the 2× Sample Buffer to room temperature. Be sure that the solution is homogenous.

- Prepare a 5 µg/µL 3X FLAG® peptide solution.
  - The 3X FLAG® peptide (N-Met-Asp-Tyr-Lys-Asp-His-Asp-Gly-Asp-Tyr-Lys-Asp-His-Asp-Ile-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C) is acidic. To dissolve it properly, add 40 µL of 10× Wash Buffer to 1 mg of 3X FLAG® peptide.
  - When the peptide is completely dissolved, add 160 µL of distilled water to the sample.
  - Mix well and store aliquots at –20 °C.
- Dilute a portion (~2 µL) of the FLAG-BAP™ fusion protein stock solution to a concentration of 50 ng/µL with 1× Wash Buffer. The diluted solution is stable at –20 °C for about two months.
- Prepare 5 mL of 1× Wash Buffer for each immunoprecipitation sample by adding 0.5 mL of 10× Wash Buffer to 4.5 mL sterile deionized water and mixing well.

Procedure

- Perform all steps at 2-8 °C, unless the procedure specifies otherwise.
- Use precooled Lysis and Wash Buffers and equipment.
- **Do not precool** the Sample and Elution Buffers.
- All centrifugations are done at 2-8 °C with precooled rotors.
- For antigens and protein:protein complexes that require a special lysis buffer composed of a different percentage of a detergent, it is recommended to use the 10× Wash Buffer as a core buffer.
- The ANTI-FLAG® M2 affinity gel is resistant to the following detergents:
  - 5.0% TWEEN® 20
  - 5.0% TRITON® X-100
  - 0.1% IGEPAL® CA-630
  - 0.1% CHAPS
  - 0.2% digitonin
- The ANTI-FLAG® M2 affinity gel can also be used with 1.0 M NaCl or 1.0 M urea. Do **not** use the ANTI-FLAG® M2 affinity gel in the presence of such reagents as:
  - SDS
  - 2-mercaptoethanol
  - Dithiothreitol (DTT)
  - Deoxycholate (DOC)
  - Guanidine HCl

  Note: this is not a comprehensive list of interfering substances.

Cell lysis

For a 70-90% confluent 100 mm dish (10^6-10^7 cells), use 1 mL Lysis Buffer. If the expression level of the FLAG® fusion protein is relatively low, lyse the cells with a reduced volume of Lysis Buffer. It is highly recommended to add protease inhibitor cocktail (Cat. No. P8340) to the Lysis Buffer (10 µL per 1 mL of Lysis Buffer), especially if the lysate is to be stored for further use.

1. Wash cells.
   a. For adherent cells:
      i. Remove the growth medium from the cells to be assayed.
      ii. Rinse the cells twice with PBS buffer, being careful not to dislodge any of the cells.
      iii. Discard PBS.
      iv. Add lysis buffer (10^6–10^7 cells/mL).
   b. For cells in suspension:
      i. Collect the cells into an appropriate centrifuge conical test tube.
      ii. Centrifuge for 5 minutes at 420 × *g*.
      iii. Decant the supernatant and discard.
      iv. Wash the cells twice by re-suspending the cell pellet with PBS.
      v. Centrifuge for 5 minutes at 420 × *g*.
      vi. Decant the supernatant and discard.
      vii. Resuspend the cell pellet in Lysis Buffer (10^6-10^7 cells/mL).
2. Incubate the cells 15-30 minutes on a shaker.
3. For adherent cells only, scrape and collect cells. For cells in suspension, proceed to Step 4.
4. Centrifuge the cell lysate for 10 minutes at 12,000 × *g*.
5. Transfer the supernatant to a chilled test tube. For immediate use, keep on ice. If the supernatant is not to be used immediately, store it at –70 °C.

FLAG® Fusion Protein Immunoprecipitation (IP)

The procedure described is for a single IP reaction. For multiple IP reactions, calculate the volume of reagents needed according to the number of samples to be processed.

For easy performance of IP reactions, it is recommended to use 40 µL of gel suspension per reaction (~20 µL of packed gel volume). Smaller amounts of resin (~10 µL of packed gel volume, which binds >1 µg FLAG® fusion protein) can be used.

Two control reactions are recommended for the procedure:

- The first control is IP with FLAG-BAP™ fusion protein (positive control).
- The second is a reagent blank with no protein (negative control).

The immunoprecipitation procedure includes several steps of precipitation and supernatant removal. To avoid resin removal during the wash and elution steps, it is possible to use a column for the IP reaction. Place an empty chromatography spin column (with a frit) in a centrifuge tube for support.

Load the resin on the column. Wash the resin using centrifugation to remove the wash solutions. If the column has an outlet plug, the binding and elution steps may be performed on the column. An efficient separation of the eluted protein from the resin is performed by a short centrifugation. This procedure is suitable for either small (20 µL bed volume) or large resin volumes.

1. Thoroughly suspend the ANTI-FLAG® M2 affinity gel in the vial, to make a uniform suspension of the resin. The ratio of suspension to packed gel volume should be 2:1. Immediately transfer 40 µL of the resin in its suspension buffer to a fresh test tube to allow a homogenous dispensation of the resin. For resin transfer, use a plastic pipette tip with the end enlarged to allow the resin to be transferred.
2. Centrifuge the resin at 5,000-8,200 × *g* for 30 seconds. To allow the resin to settle in the tube, wait for 1-2 minutes before handling the samples. Remove the supernatant with a narrow-end pipette tip or a Hamilton syringe, being careful not to transfer any resin. Narrow-end pipette tips can be made, using forceps, to pinch the opening of a plastic pipette tip until it is partially closed.
3. Wash the packed gel twice with 0.5 mL of 1× Wash Buffer. Be sure that most of the wash buffer is removed and no resin is discarded.

Optional Wash Step

To remove any traces of an unbound ANTI-FLAG® antibody from the resin suspension:

- Wash the resin with 0.5 mL Elution Buffer before continuing with the binding step.
- Do not leave the resin in Elution Buffer more than 20 minutes.
- Discard the supernatant immediately, being careful to remove all supernatant from the resin.
- Follow with three washes with 0.5 mL 1× Wash Buffer each.

If the Optional Wash Step is not performed:

- Wash the packed gel an additional 2-3 times using 0.5 mL of 1× Wash Buffer for each wash. This ensures that all of the glycerol is removed before the protein is bound to the gel.
- In case of numerous IP samples, wash the resin that is needed for all samples together.
- After washing, divide the resin according to the number of samples tested.
- Each wash should be performed with a 1× Wash Buffer at a volume equal to 20 times the total packed gel volume.

4. Add 200-1000 µL of cell lysate to the washed resin. If necessary, bring the final volume to 1 mL by adding Lysis Buffer. The volume of cell lysate to be used depends on the expression level of FLAG® fusion protein in the transfected cells.
   - For the positive control, add 1 mL 1× Wash Buffer and 4 µL of 50 ng/µL FLAG-BAP™ fusion protein (~200 ng) to the washed resin.
   - For the negative control, add only 1 mL of Lysis Buffer with **no** protein.
   - The amount of FLAG-BAP™ fusion protein to be precipitated depends on the detection method. 200 ng of protein is sufficient for an activity assay or for an immunoblot analysis.
     For SDS-PAGE analysis with Coomassie® blue or with silver staining, use 1 µg of FLAG-BAP™ fusion protein.
5. Agitate or shake all samples and controls gently for 2 hours. (A roller shaker is recommended.) To increase the binding efficiency, the binding step may be extended overnight.
6. Centrifuge the resin at 5,000-8,200 × *g* for 30 seconds. Remove the supernatants with a narrow-end pipette tip.
7. Wash the resin three times with 0.5 mL of 1× Wash Buffer. Make sure that all the supernatant is removed by using a Hamilton syringe or equivalent device.

Elution of the FLAG®-fusion protein

Three elution methods are recommended, according to protein characteristics or downstream usage:

- Protein elution under native conditions by a competition with 3X FLAG® peptide. The elution efficiency is very high with this method.
- Elution under acidic conditions with 0.1 M glycine, pH 3.5 (Elution Buffer). This is a fast and efficient elution method. Equilibration of the eluted protein with Wash Buffer may help preserve its activity.

- Elution with Sample Buffer for gel electrophoresis and immunoblotting.

Elution with 3X FLAG peptide

1. Prepare 3X FLAG® Elution Buffer. Add 3 µL of 5 µg/µL 3X FLAG® peptide solution to 100 µL of 1× Wash Buffer (150 ng/µL final concentration).
2. Add 100 µL of 3X FLAG® Elution Buffer to the resin in the test tube.
3. Incubate the samples and controls with gentle shaking for 30 minutes at 4 °C.
4. Centrifuge the resin at 5,000-8,200 × *g* for 30 seconds. Transfer the supernatants to fresh test tubes using a Hamilton syringe or equivalent device. Be careful not to transfer any resin**.**
5. Storage of supernatants:
   - For immediate use, store the supernatants at 2-8 °C.
   - For long-term storage, store the supernatants at –20 °C.

Elution with Elution Buffer

This procedure should be performed at room temperature. **Do not leave the resin in the Elution Buffer more than 20 minutes.**

1. Add 100 µL of Elution Buffer (Component Number E6150) to each sample and control resin.
2. Incubate the samples and controls with gentle shaking for 5 minutes at room temperature.
3. Centrifuge the resin at 5,000-8,200 × *g* for 30 seconds. Transfer the supernatants to fresh test tubes containing 10 µL of 10× Wash Buffer, using a Hamilton syringe or equivalent device. Be careful not to transfer any resin.
4. For immediate use, store the supernatant at 2-8 °C.
5. For long-term storage, store the supernatant at –20 °C.

Elution with Sample Buffer

The procedure should be performed at room temperature. Sample Buffer should be at room temperature before use.

To minimize the denaturation of the antibody, no reducing agent (such as 2-mercaptoethanol or DTT) is included in the Sample Buffer in this kit.

If reducing conditions are absolutely necessary, a reducing agent may be added. The final concentration of 2-mercaptoethanol or DTT in the 1× Sample Buffer should be no more than 5% or 50 mM, respectively.

1. Add 20 µL of 2× Sample Buffer to each sample and control.
2. Boil the samples and controls for 3 minutes.
3. Centrifuge the samples and controls at 5,000-8,200 × *g* for 30 seconds to pellet any undissolved agarose.
4. Transfer the supernatants to fresh test tubes with a Hamilton syringe or a narrow-end Pasteur pipette. The samples and controls are ready for loading on SDS-PAGE and immunoblotting using ANTI-FLAG® or specific antibodies against the tagged protein.

Detection of the positive control (FLAG-BAP™ fusion protein)

1. Run the samples and controls on an SDS-PAGE.
   - The protein has a molecular mass of 49.3 kDa.
   - It migrates as a 45-55 kDa band by SDS-PAGE depending on the electrophoresis conditions.
   - Immunoblot the gel with ANTI-FLAG® or anti-Bacterial Alkaline Phosphatase (BAP) antibodies.
   - Alternatively, stain the gel with a staining method such as silver staining or Coomassie blue.
2. Detect the BAP presence by enzymatic activity. SIGMA*FAST* pNPP substrate tablets set (Cat. No. N1891) or pNPP liquid substrate system (Cat. No. N7653) are recommended for the detection of BAP activity.

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Troubleshooting Guide

Problem	Possible reason	Solution
Low yield of eluted protein	Low binding efficiency	Increase the amount of lysate/protein in the binding step, and/or extend the binding duration to overnight.
	Low elution efficiency	Improve the elution in one of two ways: • Increase the concentration of 3X FLAG® Peptide in the elution solution. • Add salt to the Elution Buffer (0.1 M glycine, pH 3.5).
Appearance of multiple bands in SDS-PAGE in samples eluted with Sample Buffer	ANTI-FLAG®-M2 antibody subunits are removed from the resin by SDS or reducing agents (such as DTT, 2-mercapto-ethanol, DTE)	Use sample buffer with no SDS or reducing agents. Elute the protein by boiling. After the separation of the supernatant from the resin, SDS can be added. Then samples should be boiled and analyzed by SDS-PAGE.

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