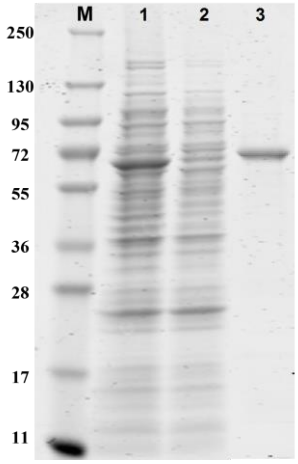
Immunoprecipitation Kit: for DYKDDDDK (FLAG®)

I. Overview

Rockland Immunochemicals' **Anti-DYKDDDDK (FLAG®) Kit for Immunoprecipitation** is intended to provide a simple, reliable and convenient purification system for recombinant proteins containing the FLAG® epitope tag. Immunoprecipitation is a powerful technique for the isolation of proteins or protein complexes. Immunoprecipitation consists of several steps including cell lysis, binding of specific antigen to an antibody, antibody-antigen complex precipitation, precipitant wash steps and the dissociation of antigen from the complex. The FLAG® epitope tag is a small but highly immunogenic peptide DYKDDDDK (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C), 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. Rockland's Anti-DYKDDDDK (FLAG®) Kit allows a rapid and efficient immunoprecipitation and elution of an active FLAG® -tagged recombinant protein in less than 2 hours. The immunoprecipitation is performed with anti-FLAG® antibody coupled to agarose beads, which are generated by covalently linking agarose to a highly specific mouse monoclonal antibody raised against FLAG®. The provided protocol is a guideline. Any procedure can be altered according to specific experimental requirements. This kit is sufficient to perform 50 X 20 µL reactions and is stable for at least 1 year when stored as indicated. Please read the entire product insert prior to use.

II. Kit Principle

Rockland's **Anti-DYKDDDDK (FLAG®)** allows for the purification by immunoprecipitation of recombinant proteins containing the FLAG® epitope tag provided by the user. The kit relies upon the high specificity of monoclonal antibody raised against the FLAG® epitope tag. This method is far easier and less costly than using antibodies produced against the recombinant protein itself therefore saving time and resources. Using the agarose bound antibody in this kit allows for efficient binding of FLAG® tag proteins without the need for preliminary steps or calibration. The immunoprecipitated FLAG® tag protein can be efficiently eluted from the agarose beads using a low pH elution step. The user is able to further characterize the resultant purified protein by size, post-translational modification, western blot and other assays.



Coomassie stained SDS-PAGE using Anti-DYKDDDDK (FLAG®) Kit for Immunoprecipitation to purify recombinant proteins containing the FLAG™ epitope tag from an E.coli cell lysate. Lane 1 – cell lysate ***before*** immunoprecipitation. Lane 2 – cell lysate ***after*** immunoprecipitation showing depletion of protein. Lane 3 – purified FLAG® -tagged recombinant protein.

III. Intended Use

Use Rockland's Anti-DYKDDDDK (FLAG®) Kit for the purification by immunoprecipitation of recombinant proteins containing the FLAG® epitope tag provided by the user. Highly active antibody binds a maximum amount of FLAG® tagged protein that is subsequently eluted from the agarose bead bound antibody by changing the buffer conditions. Non-specific proteins are first removed by copious washing steps. See "Additional Notes" for helpful tips. If you require additional assistance please call or e-mail our technical service representatives at 800-656-7625 or tech@rockland-inc.com.

IV. Storage and Stability

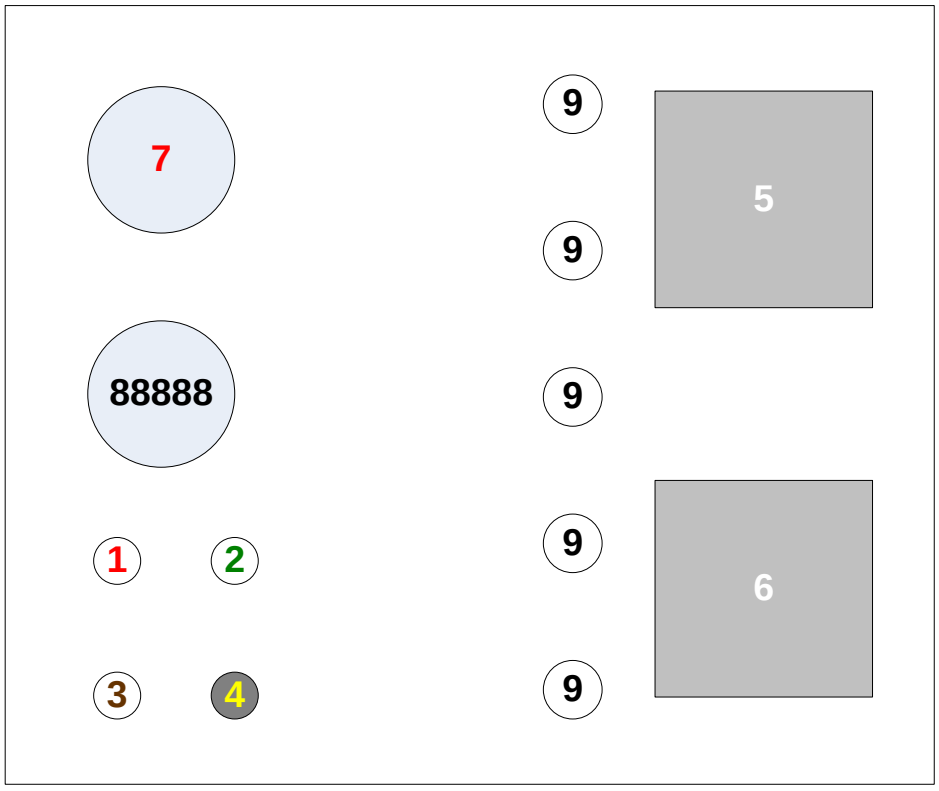
This kit is stable for at least one year when stored as indicated. Upon receipt store items 2-3 at -20°C. Store items 1 and 4-7 at 4°C. Store items 8-9 at room temperature. Individual components are stable for 3-4 weeks after dilution when stored at 4°C.

V. Number of Assays

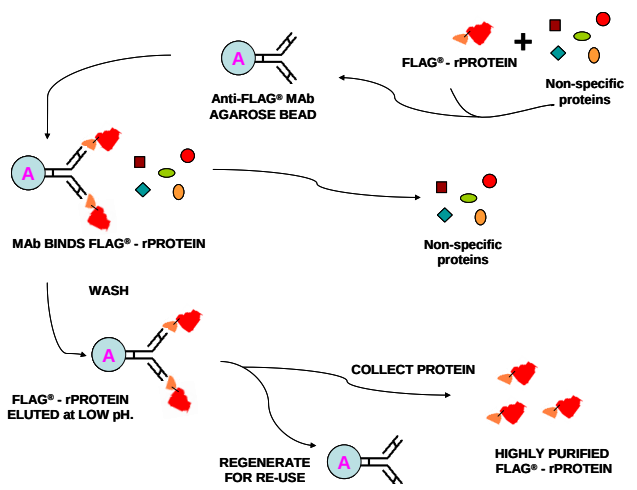
The reagents in Rockland's Anti-DYKDDDDK (FLAG®) Kit are sufficient to perform 50 X 20 µL reactions.

VI. Kit Components and Layout

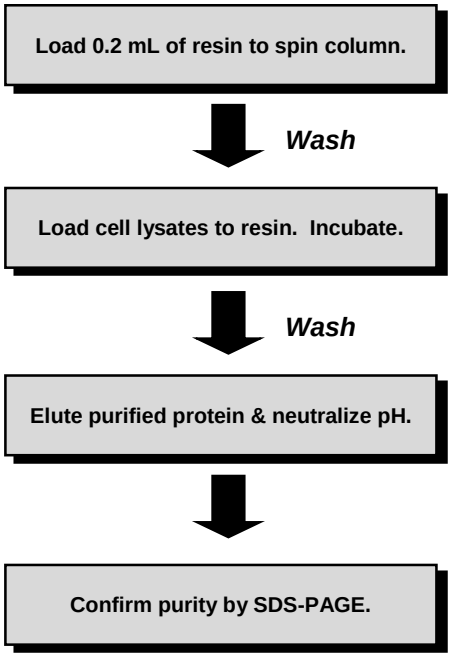
1. (1) x 1.5 mL suspension with 1 cc of **anti-FLAG® coupled to agarose** beads in clear plastic 2 mL vial with **RED** cap. Store at 4° C.
2. (1) x 0.2 mL of **FLAG® positive control lysate** in plastic 0.5 mL vial with **GREEN** cap. Store at -20° C.
3. (1) x 1.5 mL of **2X SDS-PAGE Sample Buffer** in plastic 2.0 mL vial with **AMBER** cap. Store at -20° C.
4. (1) x 2 mL of **Neutralization Buffer** in plastic 2.0 mL vial with **YELLOW** cap. Store at 4° C.
5. (1) x 50 mL of **1X Lysis Buffer** in 60 mL Nalgene® plastic bottle with **WHITE** cap. Store at 4° C.
6. (1) x 50 mL of **10X Wash Buffer** in 60 mL Nalgene® plastic bottle with **WHITE** cap. Store at 4° C.
7. (1) x 10 mL of low pH **Elution Buffer** in clear glass 10 mL vial with **RED** cap. Store at 4° C.
8. (5) x **Microspin Columns**. Store at room temperature.
9. (5) x **Collection Tubes**. Store at room temperature.
10. Instruction Manual



VII. Immunoprecipitation and Flow diagram for Anti-DYKDDDDK (FLAG®) Kit



Immunoprecipitation. Antibody binding principles are shown for the **Anti-DYKDDDDK (FLAG®) Kit** to purify FLAG® - tagged recombinant proteins. The anti-FLAG® antibody bound to agarose beads captures the FLAG® - tagged recombinant protein from solution. After copious washing with PBS, the buffer condition is changed allowing the release and collection of the highly purified recombinant protein.



VIII. Materials Required but Not Supplied

Nearly all components required for immunoprecipitation and purification of recombinant proteins containing the FLAG® epitope tag have been provided for your convenience as reagents in the Anti-DYKDDDDK (FLAG®) Kit. Some additional materials that may be needed are detailed below:

- Transfer pipettes or micro pipettes
- Microcentrifuge
- Additional tubes and/or microcentrifuge tubes with caps
- Recombinant protein or lysate containing the FLAG® epitope tag
- Protease cocktail inhibitor
- Materials relating to cell culture (if necessary)
- 0.22-um syringe filter (or equivalent)
- Liquid nitrogen (LN2)
- 18 gauge needle and syringe
- SDS-PAGE gels, equipment and related materials

IX. Preparation of Working Solutions and Lysates

- Preparation of buffers

Wash Buffer:

We have included 50 mL of 10X PBS sufficient to produce 500 mL of 1X Wash Buffer. Prepare solution using deionized water (or equivalent). Pass solutions through a 0.22-um filter prior to use. Store diluted solutions at +4°C for a maximum of 3 to 4 weeks. **This buffer does not contain preservative.** Dilute 10 mL of 10X PBS provided in the **WHITE** capped bottle up to 100 mL with deionized water. Mix thoroughly. There is no need to adjust pH. The resultant buffered saline solution is ready-to-use and contains 0.01 M Sodium Phosphate, 0.14 M Sodium Chloride, pH 7.2. Chill to 2 to 8 °C prior to use.

Lysis Buffer:

Lysis Buffer is provided as a ready-to-use 1X solution containing 50 mM Tris Cl, 150 mM Sodium Chloride, 1 mM EDTA, 1% TRITON·X-100, pH 7.4. No preparation is required prior to use. Chill to 2 to 8 °C prior to use.

- Preparation of mammalian cell lysates

Generally 5 x 10^6 to 1 x 10^7 cells are required to produce recombinant protein from mammalian cells in sufficient quantity for purification. Cells expressing the FLAG® -tagged recombinant protein of interest should be seeded and grown in the appropriate medium until they are 80-90% confluent. Harvest cells using trypsin following standard cell culture procedures. Collect cells by centrifugation. Wash the cells twice by re-suspending the cell pellet with 1X Wash Buffer and centrifuge for 5 min at 420 x *g*. Decant the supernatant and discard. Resuspend the cell pellet in Lysis Buffer at 5 x 10^6 to 1 x 10^7 cells/mL. The cell lysates can be frozen in liquid nitrogen and stored at -70°C prior to use. If the expression level of the FLAG® -tagged recombinant protein is relatively low, then lyse the cells at a higher concentration of cells/mL to increase the concentration of recombinant protein. It is highly recommended to add a protease inhibitor cocktail to the lysis buffer especially if stored for future use.

1. Resuspend the cell pellet in **Lysis Buffer** at 5 x 10^6 to 1 x 10^7 cells/mL. Add sufficient amount of protease inhibitor cocktail to the Lysis Buffer.
2. Lyse the cells by two **freeze-thaw cycles** using liquid nitrogen. Alternatively a dry ice/ethanol bath followed by immersion in a 42 °C water bath may be used.
3. **Shear** DNA by passing the preparation through an 18-gauge needle 3 – 4 times.
4. **Centrifuge** the cell lysate for 10 min at 12,000 rpm to pellet the cellular debris. Transfer supernatant to a fresh tube.
5. **Maintain** on ice for immediate use or store at -70 °C for future use.

- Preparation of Cell Lysates from other sources

Researchers should follow appropriate protocols when preparing cell lysates from bacteria, yeast, and insect or plant cells. Some detergents such as SDS, 2-mercaptoethanol, dithiothreitol (DTT), deoxycholate (DOC) or chaotropes like guanidine HCl may reduce the affinity of anti-FLAG® antibody for FLAG® -tagged recombinant proteins. Researchers should determine the optimum cell lysis buffer conditions before performing the intended experiment.

X. Immunoprecipitation Method

The following method is suggested as a **guideline** for the use of Rockland's Anti-DYKDDDDK (FLAG®) Kit. The highly active antibody bound to agarose beads in this kit binds a maximum amount of FLAG® tagged protein that is subsequently eluted by changing the buffer conditions. The kit is simple and easy to use. Depending on the nature of the user's lysates or recombinant protein, specific conditions may be changed as necessary. All reactions can be performed at 2 to 8 °C unless stated otherwise. Remember to use cold Wash Buffer and Lysis Buffer and to pre-cool centrifuges. See the troubleshooting guide for recommendations to use the **FLAG® positive control lysate** if necessary.

Preparing the Microspin Column for Use:

1. Snap off the tip of a **microspin column** and add 0.2 mL of **anti-FLAG® coupled to agarose** beads as a slurry into the top part of the column. Note: as little as 20 µL agarose beads can be used per reaction.
2. Place the microspin column into a **collection tube** and centrifuge for 1 minute in a microcentrifuge pre-cooled to 2 to 8 °C.
3. Remove the microspin column from the collection tube and discard the flow-through buffer. Place the spin column back into the same **collection tube**.
4. Wash the agarose beads by adding 0.4 mL of **1X Wash Buffer** to the microspin column. Centrifuge as before.
5. Remove the microspin column from the collection tube and again discard the flow-through buffer. Place the spin column back into the same **collection tube**.
6. **Repeat** steps 4 and 5 two (2) more times for a total of three (3) washes.

Loading the Sample:

1. Load 0.2 to 0.5 mL of the **sample of cell lysate** containing the FLAG® -tagged recombinant protein to be purified to the agarose in the microspin column.
2. **Incubate** for 30 min to 1 h at room temperature. Some FLAG® -tagged recombinant proteins may require incubation overnight at 4°C for maximum binding.
3. **Centrifuge** for 1 min in a microcentrifuge. Steps 1 and 2 may be repeated up to two (2) additional times for larger volume samples until the entire sample has been applied. Be careful not to exceed the binding capacity of the agarose (0.6 mg FLAG® -tagged recombinant protein/cc agarose). Save the flow through solution from sample loading in a microcentrifuge tube for future analysis, if desired.
4. **Wash** the agarose beads to remove non-specific proteins by adding 0.6 mL of 1X Wash Buffer.
5. **Centrifuge** for 1 minute in a microcentrifuge and discard the flow-through buffer.
6. **Repeat** steps 4 and 5 three (3) more times for a total of four (4) washes.

Eluting the purified recombinant protein:

1. **Transfer** the microspin column to a clean microcentrifuge tube with cap.
2. Add 0.1 mL to 0.2 mL of **Elution Buffer** to the microspin column. Using a pipette, suspend the anti-FLAG® coupled to agarose beads in the Elution Buffer.
3. **Incubate** 5 min at room temperature. No further agitation is required (see additional notes).

4. **Centrifuge** as before to collect the eluate (i.e. FLAG® -tagged recombinant protein purified from sample).
5. *Immediately* neutralize the eluate with 15 µL of **Neutralization Buffer**.
6. Regenerate the resins by **repeating washing** with 1X Wash Buffer for re-use.
7. For **storage** suspend the anti-FLAG® coupled to agarose beads in 1X Wash Buffer and add glycerol to 50%. A preservative may be added prior to storage at -20° C.

Post-purification analysis of the FLAG® -tagged recombinant protein:

1. **Prepare** SDS-PAGE apparatus, gel and buffers for protein electrophoresis according to standard procedures. Include molecular weight markers for size comparison to purified FLAG® -tagged recombinant protein. Optional: include varying amounts of known control protein to create a standard curve for estimation of yield of your purified FLAG® -tagged recombinant protein.
2. **Transfer** aliquots containing a sufficient amount of neutralized purified FLAG® -tagged recombinant protein, unpurified lysate and the flow through solution (collected after sample loading) separately into clean microcentrifuge tubes with caps.
3. Add an equal volume of **2X SDS-PAGE Sample Buffer** to each sample. Add reducing agent if desired to each sample. Boil samples prior to loading.
4. Load samples to gel. Connect electrodes. **Separate by SDS-PAGE** according to standard procedures.
5. Stain and destain gel for visualization. **Document results** and analyze the efficiency of purification by comparison to control proteins.

XI. Additional Notes

- The method given in these instructions is to be used as a guideline. Experienced users can make deviations from the outlined procedure. Note that the solutions have been optimized for the given method and alteration of the reagent concentrations, volumes, reaction times, or temperature will affect the overall performance of the kit. Generally, when modifying conditions experimentally, only alter one variable at a time.
- The anti-FLAG® coupled to agarose beads can withstand forces up to 5,000 x rpm without collapsing.
- The sample of cell lysate containing the FLAG® -tagged recombinant protein should be in phosphate buffered saline (PBS) or any other physiological buffer at or near neutral pH. The flow-through from sample loading is recommended to be saved and analyzed for comparison with starting material and eluted purified recombinant protein to ensure complete isolation of the fusion protein.
- **Do not** allow the anti-FLAG® coupled to agarose beads to remain in low pH elution buffer for longer than the stated time. Do not exceed more than 15 min of total contact time at low pH. Excessive exposure to low pH may inactivate the antibody preventing the regeneration and re-use of the agarose beads.
- Certain antigens and/or protein complexes may require a special lysis buffer composed of a different percentage of detergent. PBS is strongly recommended as a core buffer in any lysis buffer used in advanced applications of this kit. Alternative conditions must be determined empirically by the user. The anti-FLAG® antibody coupled to agarose beads is resistant to the following detergents: 5.0% TWEEN-20, 5.0% TRITON X-100, 0.1% CHAPS and 0.2% digitonin as well as 1.0 M sodium chloride and 1.0 M urea.

- Never use the anti-FLAG® coupled to agarose beads with any concentration of sodium dodecyl sulfate (SDS), 2-mercaptoethanol (ßME), dithiothreitol (DTT), deoxycholate (DOC) or guanidine HCl. This is not a comprehensive list of interferences.
- The anti-FLAG® agarose bead resin can be reused up to three (3) times without loss of binding capacity when properly handled and stored. Spin columns also can be reused after sanitization and removal of nucleic acids and proteins using 0.1M NaOH.
- Store the components of this kit as indicated on page 2.
- The individual components of this kit may be ordered separately.

XII. Troubleshooting Guide

Problem: Low yield of eluted protein.
Cause: Low binding efficiency, low elution efficiency or low protein expression
Solution: Increase the amount of lysate/protein in the binding step and/or extend the incubation time to overnight at 4°C. Use the provided **FLAG® positive control lysate** as a control in all experiments.
Repeat the elution step making certain that the agarose beads are well suspended in elution buffer prior to centrifugation.
Verify protein expression by SDS-PAGE gel or western blotting prior to performing Anti-DYKDDDDK (FLAG®) immunoprecipitation. Use **HRP MAb for the detection of FLAG®** for western blotting.

Problem: Appearance of multiple bands by SDS-PAGE in eluted samples.
Cause: Washing step was not sufficient to eliminate non-specific binding
Solution: Increase the number of washing steps after the sample is loaded onto the agarose beads. Consider a more stringent washing buffer such as PBST (see Section XI - Additional Notes).
Cause: FLAG® -tagged recombinant protein expressed as multiple forms with varying molecular weights.
Solution: Verify expression by Western blot. Use **HRP MAb for the detection of FLAG®** for western blotting.

XIII. References

Antibodies, A Laboratory Manual. Ed Harlow and David Lane, eds. Cold Spring Harbor Press, 1988.

Brizzard BL, Chubet RG, Vizard DL.(1994) Immunoaffinity purification of FLAG epitope-tagged bacterial alkaline phosphatase using a novel monoclonal antibody and peptide elution. *Biotechniques* **4**:730-5.

Chiang CM, Roeder RG. (1993) Expression and purification of general transcription factors by FLAG epitope-tagging and peptide elution. *Pept Res*. **6**(2):62-4.

Knappik A, Plückthun A. (1994) An improved affinity tag based on the FLAG peptide for the detection and purification of recombinant antibody fragments. *Biotechniques* **4**:754-61.

XIV. Trademarks

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XV. Disclaimer

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XVI. Additional Products and Services

Related Products for Anti-DYKDDDDK (FLAG®) Kit for Immunoprecipitation

Product	Catalog No.	Size
Antibody for the detection of FLAG™ (MOUSE) MAb - Agarose Conjugate	200-350-383	1 cc
2X SDS-PAGE Sample Buffer	MB-018	100 mL
1X RIPA Lysis Buffer	MB-030-0050	50 mL
10X PBS	MB-008	1.0 L
10X PBST	MB-075-1000	1.0 L