Anti-DDK Magnetic Immunoprecipitation Kit

Catalog # AR100024

Table of Contents

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Immunoprecipitation is a technique designed to affinity purify proteins or protein complexes through the use of a highly specific antibody. This technique can successfully concentrate or precipitate a particular protein from a cell lysate or mixture of proteins. It is one of the most widely used molecular biology methodologies to investigate protein activation, protein post-translational modifications, and protein-protein interactions or co-immunoprecipitation.

OriGene's highly specific anti-DDK mouse monoclonal antibody (4C5) recognizes the DYKDDDDK epitope tag which is the same as the Sigma Flag™ tag. The 4C5 antibody is covalently coupled to magnetic beads and has been validated for use in immunoprecipitation and co-immunoprecipitation experiments. For efficient binding with the anti-DDK magnetic beads, the target protein must contain a **carboxy-terminus** fused DDK epitope tag. For expression and immunoprecipitation of human proteins we recommend the use of cDNA clones from OriGene's TrueORF™ clone collection which all contain the appropriate DDK epitope tag. Alternatively, OriGene can provide a custom clone containing the appropriate tag or DDK-tagged proteins can be immunoprecipitated directly from any of OriGene's >17,000 human over-expression lysates.

The kit also includes Control Beads that have been coupled to non-immune mouse IgG. The control beads can be used to pre-clear the sample by binding to proteins that may bind non-specifically to mouse IgG. The control beads may also be used as a direct comparison to the anti-DDK beads in order to demonstrate that the immunoprecipitation is specific for the epitope tag.

Materials Supplied:

Description	Amount
Anti-DDK Magnetic Beads	1.25 mL
Control (mouse IgG) Magnetic Beads	1.25 mL
1X Lysis Buffer	250 mL
10X Wash Buffer	50 mL
2X SDS-PAGE Sample Buffer (nonreducing)	1.5 mL
BSA-DDK Control Protein (1 mg/ml)	50 µL

Storage of reagents:

The Immunoprecipitation Kit is shipped on blue ice. Upon receipt, the BSA-DDK control protein should be stored at -20ºC until use. All other reagents supplied in the Immunoprecipitation Kit should be stored at 2-8ºC.

Materials Required but Not Supplied Kit:

Description	Recommended Source
Protease Inhibitor Cocktail	Sigma #P2714 or equivalent.
Dithiothreitol (DTT)	Sigma #646563 or equivalent.
β-mercaptoethanol	Sigma #M3148 or equivalent.
Magnetic Separation Stand	Thermo Fisher Scientific #PI-21359 or equivalent.

Materials Recommended for Western Blot Analysis:

Description	Recommended Source
4C5 anti-DDK, peroxidase conjugate	OriGene Technologies Inc, TA150030
Nitrocellulose or PVDF Membrane	Bioexpress, F-3139-3 or F-3112-30
Luminol Reagent	OriGene Technologies Inc, TA10016
Myc/DDK tagged Molecular Weight Markers	OriGene Technologies Inc, MWM1001

Cell Lysate Production

1. Transfect a 50-70% confluent monolayer of cells with plasmid DNA using an appropriate transfection agent. For expression of human proteins in HEK293 cells we recommend the use of OriGene's TrueORF™ clones in combination with Turbofectin 8.0 transfection reagent. Optimal transfection results for specific cell types should be experimentally determined by the end user. Alternatively, proteins can be immunoprecipitated from over 17,000 human over-expression lysates offered by OriGene.
2. Incubate transfected cells for 24-48 hours.
3. Remove cell culture media supernatant from culture dish. Wash cells with sterile 1X PBS, pH 7.4 to completely remove all media from the culture dish.
4. Prepare 1X Lysis Buffer containing 1X Protease Inhibitors (see Preparation of Materials and Buffers, below). Add 1ml of 1X Lysis Buffer per 100 mm petri dish (10^6 – 10^7) cells.
5. Pipette up and down or agitate using a cell scraper to thoroughly remove all cells from the culture dish. Add the cell lysis buffer containing removed cells to a 1.5 ml Eppendorf tube.
6. Incubate on ice for 20 minutes.
7. Clear the cell lysate by centrifugation at 12,000 – 14,000 x g for 5 minutes.
8. Transfer the cell lysate supernatant to a new 1.5 ml Eppendorf tube. Store cell lysate at -70ºC to -80ºC until ready for use.

Immunoprecipitation Protocol

The following protocol is for a single immunoprecipitation reaction. For multiple individual immunoprecipitation reactions or bulk reactions, adjust reagent volumes according to the number of experimental samples to be processed. The optimal working conditions should be determined by the end user.

Preparation of Materials and Buffers

1. Prepare Wash Buffer by diluting 10X Wash Buffer to 1X with deionized water. Store diluted wash buffer at 2-8ºC for up to one month.

2. Prepare a 100X Protease Inhibitor Stock by dissolving the contents of one vial (Sigma #P2714) in 1 mL of sterile deionized water. The 100X Protease Inhibitor Stock should be stored at -20ºC until ready for use.

3. Prepare 1X Lysis Buffer containing 1X Protease Inhibitors. Store 1X Lysis Buffer with Protease Inhibitors at 2-8ºC for up to one month.

4. For a positive control, add 10 µl of BSA-DDK control protein to 490 µl of 1X Lysis Buffer containing protease inhibitors. Use this mixture in the immunoprecipitation below.

5. Prepare 2X SDS-PAGE Sample Buffer (nonreducing) by incubating at 37C for 10 minutes to completely thaw. To make a reducing buffer, add a reducing agent to a final concentration of 0.1M Dithiothreitol (DTT) or 5% β-mercaptoethanol.

Detailed Immunoprecipitation Procedure

Optional Pre-clear Procedure:

1. Pipette 25 µl of Control Magnetic Beads into 1 ml of 1X Lysis Buffer and mix thoroughly.
2. Place the sample onto a magnetic separation stand for one minute to magnetically separate the beads. Discard supernatant.
3. Prepare the sample by diluting 50 µl of cell lysate with 450 µl of 1X Lysis Buffer. Add the lysate or protein to the tube containing the washed magnetic beads.
4. Place the mixture on a rotator and incubate at room temperature for 1 - 2 hours.
5. Place the sample onto a magnet separation stand for one minute to magnetically separate the beads.
6. Remove supernatant for use in the Immunoprecipitation Procedure.

Immunoprecipitation Procedure:

1. Pipette 25 µl of anti-DDK magnetic beads into 1 ml of 1X Lysis Buffer and mix thoroughly.
2. Place the sample onto a magnetic separation stand for one minute to magnetically separate the beads. Discard supernatant.
3. Prepare the sample by diluting 50 µl of cell lysate with 450 µl of 1X Lysis Buffer. This step is not necessary if using pre-cleared supernatant from above.
4. Add the pre-cleared supernatant, prepared lysate or diluted protein to the tube containing the washed anti-DDK magnetic beads.
5. Place the mixture on a rotator and incubate at 2-8ºC for 2 hours to overnight.
6. Place the sample onto a magnetic separation stand for one minute to magnetically separate the beads. Remove the supernatant (the flow-through) and save for analysis.
7. Wash magnetic beads three times with 1 ml of 1X Wash Buffer. Each time, place the sample onto a magnet stand for one minute to magnetically separate the beads. Carefully remove the supernatant after each wash.
8. After the final wash, add 25 µl of 2X SDS-PAGE Sample Buffer to the magnetic beads and mix by pipetting up and down. Store the sample at -70ºC to -80ºC until Western Blot analysis. In most cases, separation of buffer from beads is not critical however the sample storage should be determined by the end user.

Immunoprecipitation Controls

Recommended Controls	Effect
BSA-DDK Protein Control	The DDK tagged BSA protein can be used as a **positive** control.
No Protein Control	No protein added to the Lysis Buffer can be used as a **negative** control.
Positive Lysate Control	A different cell lysate containing a DDK tag at the carboxy-terminus can be used as a **positive** control.
Negative Lysate Control	A negative cell lysate (i.e. GFP transfected cells or other overexpression lysate) can be used as a **negative** control.
Control Magnetic Beads	Non-immune Mouse IgG control magnetic beads are supplied in the kit as a **negative** control for the immunoprecipitation and for pre-clearing the sample.

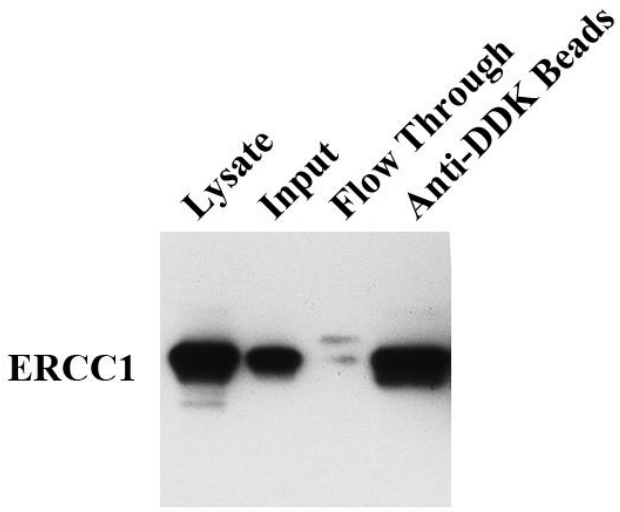
Western Blot Analysis:

For detection of immunoprecipitated proteins, we recommend using the 4C5 anti-DDK, peroxidase conjugate (OriGene, TA150030) for an easy, highly sensitive one step detection reagent for Western Blotting. The 4C5 anti-DDK, peroxidase conjugate eliminates the need for secondary antibodies that can potentially cross-react with non-specific proteins in the sample and allows for direct detection of the epitope-tagged protein with minimal background.

For co-immunoprecipitation, use a gene-specific antibody as the primary antibody in the Western Blot, followed by a species-specific HRP conjugate. The gene specific antibody will recognize the target protein if it is bound to the DDK-tagged protein in the sample. This powerful technique can be used for the discovery of specific protein-protein interactions.

Immunoprecipitation Example

Human ERCC1-Myc/DDK plasmid (OriGene Cat# RC200478) was transiently transfected into HEK293T cells. Cell lysate preparation and immunoprecipitation were performed using the above protocol. Western Blot detection was performed using the 4C5 anti-DDK, peroxidase conjugate (OriGene, TA150030). DDK-tagged ERCC1 protein was detected in the undiluted lysate, the diluted input sample, and the anti-DDK immunoprecipitated. Only a trace of ERCC1 protein is detected in the flow-through indicating that nearly 100% of the tagged protein was captured by the anti-DDK beads.



Troubleshooting

Problem	Cause	Solution
Low Expression of tagged protein in cell lysate	Clone has wrong DNA sequence	Sequence your clone to ensure there are no mutations and that the DDK epitope tag is in frame with the gene coding sequence. The stop codon must be removed.
	Clone is poorly expressed and/or toxic	Reduce the volume of 1X Lysis Buffer used for cell lysis after transfection.
	Transfection did not work	The DNA-to-Transfection Reagent ratio needs to be optimized. See manufacturer's instructions for cell transfection troubleshooting.
Small magnetic bead pellet	Magnetic beads not prepared correctly	Pipette magnetic beads up and down to prevent bead settling. Beads should be homogenous before use.
	Magnetic beads lost during washes	Pipette carefully to discard supernatant after each wash. Do not disrupt bead pellet during this step. Each IP sample should be magnetically separated on the magnet stand for a minimum of one minute to ensure beads are separated.
Western blot has failed	Western blot problems	Here is an example Western Blot Protocol.
Nonspecific bands present in SDS-PAGE gel or Western blot	Cell lysate samples not pre-cleared	Pre-clear your IP samples by incubating each cell lysate with Control Magnetic Beads (supplied in Kit) for 1-24 hours at room temperature.

Problem	Cause	Solution
	Magnetic beads need to be pre-blocked	Pre-block the anti-DDK magnetic beads in 5% BSA or non-fat milk prior to incubation with your pre-cleared sample.
	Magnetic beads not thoroughly washed	Wash magnetic beads once in 1X Lysis Buffer to remove storage buffer before use. Wash magnetic beads three times in 1X Wash Buffer before eluting in SDS-PAGE Sample Buffer for gel analysis.
	Magnetic beads loaded into SDS-PAGE gel	After boiling your IP sample in SDS-PAGE Sample Buffer, magnetically separate on a magnetic stand. This will minimize bead loading into your SDS-PAGE gel.
	Western blot membrane not thoroughly washed.	Here is an example Western Blot Protocol. If the nonspecific bands are still present, do a high salt wash (1X Wash Buffer containing 500mM NaCl) for 5 minutes prior to developing.

Frequently Asked Questions

Q1: Can anti-DDK magnetic beads be used for purifying DDK-tagged proteins?

A1: Yes, but we recommend the magnetic beads be used only for purposes of immunoprecipitation and co-immunoprecipitation.

Q2: How much beads should I use for my immunoprecipitation experiments?

A2: We recommend using 25µl of beads per immunoprecipitation sample. However, the optimal amount of beads is dependent upon the cell type and the protein expression level. The optimal beads volume should be determined experimentally by the end user.

Q3: What are the advantages of using OriGene's anti-DDK magnetic beads compared to Flag™ magnetic beads?

A3: Our anti-DDK magnetic beads have been optimized to work with all of our 12,500 TrueORF™ Gold premium cDNA clones. Our TrueORF™ Gold clones contain the DDK epitope tag fused at the carboxy-terminus allowing for easy detection and immunprecipitation of any gene in the human genome. Additionally, our anti-DDK magnetic beads are a superior tool for the discovery of protein-protein interactions by co-immunoprecipitation.