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Isolation of epitope-tagged proteins using MACSflex™ MicroBeads

Contents

1. Description

1.1 Background information

An epitope (also called an antigenic determinant) is any structure or sequence that is recognized by the adaptive part of the immune system, for example an antibody. When such an epitope is introduced into a protein, this protein is described as being "epitope-tagged" and can be detected by an epitope specific antibody.[1,2]

It is important that the addition of a tag sequence does not affect the function, intracellular transport, modification, or location of the target protein. The use of epitope tags of only 6–20 amino acids, combined with introducing the epitope at the amino- or carboxyl-terminus of the protein, normally allows successful tagging of most proteins without loss of function.

Tags are normally added to a protein of interest by cloning the gene into commercially available expression vectors that contain one or more tag sequences. These vectors can then be introduced into a cell and the expression, localisation, and function of the tagged protein can be studied.

Epitope tagging of proteins is especially useful when no protein specific monoclonal antibodies can be made, for example, for the study of highly conserved proteins, isoforms of a gene family, splice variants, or post-translational modifications of a protein. In addition, because the interactions between the epitope tag and the monoclonal antibodies are specific and are of high affinity, it makes it much easier to optimize conditions for the isolation of the tagged protein, its interaction partners or of whole complexes. In this way the expression, screening, and characterization of new gene sequences can be greatly simplified.

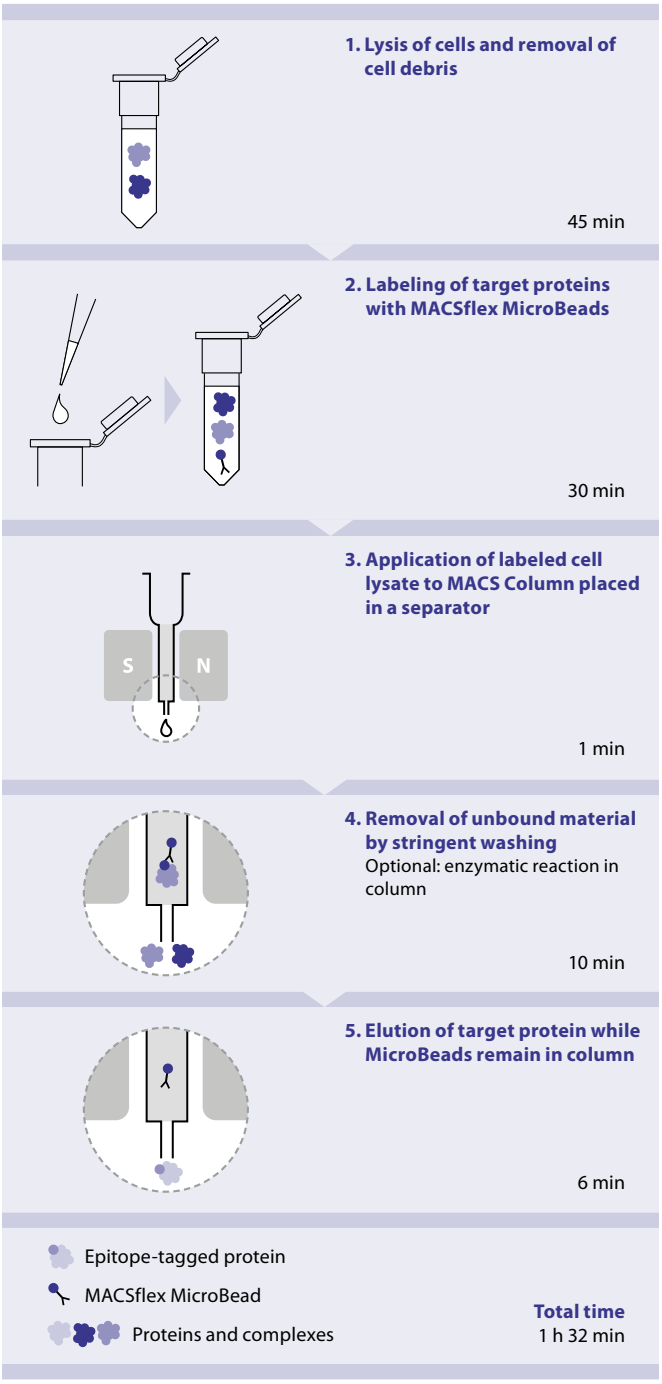

Figure 1: Overview

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Miltenyi Biotec GmbH
Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany
Phone +49 2204 8306-0, Fax +49 2204 85197
macs@miltenyibiotec.de
www.miltenyibiotec.com

Miltenyi Biotec Inc.
2303 Lindbergh Street, Auburn, CA 95602, USA
Phone 800 FOR MACS, +1 530 888 8871, Fax +1 530 888 8925
macs@miltenyibiotec.com

1.2 MACS® Technology for tagged proteins

The antibody-conjugated MACSflex™ MicroBeads bind specifically to the epitope of the target protein. The magnetically-labeled proteins are retained on a µ Column placed in the magnetic field of a µMACS™ Separator. Stringent washing steps can easily be applied to remove non-specific interacting molecules. The target molecules can then be eluted with high purity by using an Elution Buffer for SDS-PAGE; the eluate is then ready for direct SDS-PAGE analysis.
For downstream assays that require a functional, native protein, it is also possible to leave the target protein bound within the column. A solid phase enzymatic assay can then be carried out during which the MACS® Column Technology permits efficient substrate/enzyme exchange and wash steps.

For an overview and a general working scheme refer to figure 1.

1.2 Reagent and instrument requirements

- µMACS Separator (# 130-042-602) or thermoMACS™ Separator (# 130-091-136) and MACS MultiStand (# 130-042-303)
- µ Columns (#130-042-701)
- Antibody-conjugated MACSflex MicroBeads 0.5 mg (# 130-105-806) or 2.0 mg (# 130-105-805)
- Heated block (95 °C)
- Lysis buffer: 150 mM NaCl, 1% Triton® X-100, 50 mM Tris HCl (pH 8.0)
- Wash buffer 1: 150 mM NaCl, 1% Igepal® CA-630 (formerly NP-40), 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl (pH 8.0)
- Wash buffer 2: 20 mM Tris HCl (pH 7.5)
- Elution buffer for SDS-PAGE: 50 mM Tris HCl (pH 6.8), 50 mM DTT, 1% SDS, 1 mM EDTA, 0.005% bromphenol blue, 10% glycerol
- Protease inhibitors (refer to 5. Appendix)

2. General protocol

2.1 Before starting

▲ To prevent protein degradation, it is best to perform the lysis on ice. Proteinase inhibitors should be added to the lysis buffer. For details, refer to a list with appropriate protease inhibitors and effective concentrations in the section 5. Appendix, 5.1.

▲ The lysis is the most crucial step during an immunoprecipitation. The supplied lysis buffer works for a wide range of protein sources.

For special applications the supplied lysis buffer can be replaced by a lysis buffer, which is adapted to your special needs. The lysis buffer must not impair the antigen-antibody binding. Therefore, the lysis buffer conditions must be carefully chosen. Factors such as ionic strength, the pH, the concentration and type of detergent, the presence of divalent cations, co-factors, and stabilizing ligands all influence the effectiveness of a lysis buffer. Generally, the lysis buffer should not contain SDS as it may disrupt the cell nuclei (refer also to section 3.4).

▲ The lysis of entire cells results only in the partial release of nuclear proteins. In order to obtain all nuclear proteins, we recommend lysing purified nuclei. For a protocol for nuclear extract preparation, refer to section 5. Appendix, 5.2.

▲ For lysis of yeast cells or bacteria we refer to any of the common protocols found in the literature.[1]

▲ In initial experiments the same buffer that was used for the lysis of the cells should be used for column washes. In order to reduce the background of unspecifically bound proteins, a more stringent wash buffer can be chosen for subsequent experiments, e.g. wash buffer 1. Co-immunoprecipitates are much more sensitive to stringent wash buffers, therefore we recommend to use only lysis buffer for all column washes.

▲ For subsequent enzymatic reaction on the µ Column: use suitable buffers and solutions for the enzymatic reaction which is to be performed.

2.2 Lysis of cells

▲ Pre-cool appropriate lysis buffer and centrifuge to 4 °C. Prepare ice bucket.

Lysis of adherent cells

1. Remove medium from culture dish.
2. Add 1 mL pre-cooled (4 °C) lysis buffer to a 9 cm culture dish containing 1–10×10^6 cells. Scrape the lysate from the culture dish using a cell scraper and transfer to a 1.5 mL tube. Mix well and incubate for 30 minutes on ice with occasional mixing.
3. Centrifuge for 10 minutes at 10,000×g at 4 °C to sediment the cell debris.
4. Transfer the supernatant to a fresh 1.5 mL tube and proceed to magnetic labeling and separation (2.3).
5. (Optional) The lysate can also be stored at this step at –20 °C or –70 °C. Thawing should be done on ice.

Lysis of suspension cells

1. Transfer the cells of one 9 cm culture dish containing 1–10×10^6 cells to a centrifugation tube and centrifuge for 5 minutes at 300×g at 4 °C.
2. Remove supernatant and place the tube containing the cell pellet on ice. Add 1 mL of pre-cooled (4 °C) lysis buffer and mix well.
3. Incubate on ice for 30 minutes with occasional mixing.
4. Centrifuge for 10 minutes at 10,000×g at 4 °C to sediment the cell debris.
5. Transfer the supernatant to a fresh 1.5 mL tube and proceed to magnetic labeling and separation (2.3).
6. (Optional) The lysate can also be stored at this step at –20 °C or at –70 °C. Thawing should be done on ice.

2.3 Magnetic labeling and separation for subsequent analysis by SDS-PAGE

▲ Pre-heat a heated block to 95 °C.

1. Add 50 µL antibody-conjugated MACSflex MicroBeads to the lysate to magnetically label the epitope-tagged target protein. Mix well.
2. Incubate for 30 minutes on ice.

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3. Place the µ Column in the magnetic field of the µMACS Separator. Prepare the µ Column by applying 200 µL lysis buffer on the column.
4. Pipette elution buffer (80 µL for each separation) into a fresh tube and place it in the pre-heated 95 °C block.
5. After the labeling incubation has finished apply the cell lysate onto the column and let the lysate run through. Columns are "flow stop" and do not run dry.
6. Rinse column with 4×200 µL of wash buffer 1 or any other suitable buffer.
7. Rinse column with 1×100 µL wash buffer 2.

▲ **Note:** It is important that high concentrations of residual salt and detergent are removed from the immune complex prior to elution as both may interfere with a subsequent SDS-PAGE analysis.

8. Apply 20 µL of pre-heated 95 °C hot elution buffer to the column and incubate for 5 minutes at room temperature.
9. Apply 50 µL of pre-heated 95 °C hot elution buffer to the column and collect eluate as the immunoprecipitate which can now be analyzed by SDS-PAGE.

3. Tips for special applications

3.1 Lysis buffers for different protein sources

- Bacterial cell lysis: the supplied lysis buffer can be used to prepare a bacterial cell lysate by sonication.
- Yeast cell lysis: the supplied lysis buffer can be used to prepare a yeast cell lysate using glass bead disruption of the cell wall.
- In case of co-immunoprecipitations with very weak protein-protein interactions we recommend using a low salt lysis buffer: 1% Igepal CA-630 (formerly NP-40), 50 mM Tris HCl (pH 8.0).
- If strong background ionic interactions are expected, we recommend using a high salt lysis buffer : 500 mM NaCl, 1% Igepal CA-630 (formerly NP-40), 50 mM Tris HCl (pH 8.0); refer also to section 3.4.

3.2 Native protein elution

After the wash with wash buffer 2 (section 2.3, step 7), a non-denaturing elution of the column bound antigen is also possible: either by using a pH shift or by eluting the antigen–MACSflex MicroBead–complex.

Elution by pH shift using triethylamine, pH 11.8

Proceed after 2.3, step 7:

▲ Rinse µ Column with 1×100 µL wash buffer 2.

1. Apply 20 µL of 0.1 M triethylamine, pH 11.8, 0.1% Triton X-100 to the column and incubate for 5 minutes at room temperature.
2. Apply 50 µL of 0.1 M triethylamine, pH 11.8, 0.1% Triton X-100 and collect eluate in a tube containing 3 µL of 1 M MES, pH 3 for neutralisation.
3. Repeat last step twice, collecting the eluates in separate fractions and analyse the eluates by SDS-PAGE and/or Western blot or use in downstream assays.

Elution of antigen–MACSflex MicroBead–complex

Proceed after 2.3, step 7:

▲ Rinse µ Column with 1×100 µL wash buffer 2.

1. Remove the µ Column from the µMACS Separator.
2. Apply 50 µL of a suitable buffer to the column and collect the brown eluate.
3. Repeat last step once. The eluates can now be used in downstream assays.

3.3 Enzymatic reactions on the column

Enzymatic reactions with the immunoprecipitated epitope-tagged protein complex can be carried out while the protein remains bound to the µ Column. Performing the reaction on the µ Column offers the advantage of very convenient handling, especially when working with radioactively labeled proteins or substrates. It also allows a serial enzymatic reaction to be performed on the same µ Column.
A few guidelines are listed below on how to perform the enzymatic reaction on the µ Column, however, every enzymatic reaction must be performed with different optimised conditions.

▲ The lysis of the cells and the magnetic labeling should be performed as described in section 2.3, Lysis of cells and 2.4, Magnetic labeling and separation for subsequent analysis by SDS-PAGE. However, neither lysis nor wash buffer should contain SDS since it may impair the biological activity of the immunoprecipitated complex. The magnetic separation conditions, e.g., type of wash buffer and number of wash steps, should be chosen so that non-specific proteins are efficiently removed. Prior to the enzymatic reaction, the µ Column should be rinsed with 1.100 µL of reaction buffer used for the enzymatic reaction.

▲ The void volume of the µ Column is 25 µL. Thus, buffers and solutions used for incubation with the immobilized immunoprecipitate should always be applied in 25 µL aliquots. If it is necessary to incubate immobilized immunoprecipitate with a volume >25 µL, sequentially incubate in steps of 25 µL aliquots until the total volume has been applied.

▲ For incubation at 37 °C and 42 °C the thermoMACS should be used. For different incubation temperatures the column and µMACS Separator should be placed in an incubator set at the appropriate temperature.

▲ After the enzymatic reaction has been performed, the immunoprecipitated protein (complexes) can be eluted for SDS-PAGE analysis. Therefore, remove residual salt by rinsing the µ Column with 2×100 µL wash buffer 2. Then, apply 20 µL hot (95 °C) elution buffer to the column and incubate for 5 minutes at room temperature. Apply 50 µL hot (95 °C) elution buffer and collect eluate as the immunoprecipitated target protein. The target protein (complexes) can now be analyzed by SDS-PAGE.

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3.4 Tips & hints

The function of different buffer components:

Detergents – these are partially hydrophobic and partially hydrophilic and can solubilize membranes and membrane proteins. They work to increase protein solubility and decrease aggregation. Non-ionic detergents tend to be more gentle in their actions than ionic detergents and are more suitable for protein-protein interaction studies.

Salts – increasing the salt concentration in the buffer will decrease ionic interactions between proteins in a cell lysate.

▲ **Note:** To create a high salt lysis buffer, add 0.2 g NaCl per 10 mL lysis buffer.

▲ **Note:** To create a high salt wash buffer, add 0.2 g NaCl per 10 mL wash buffer.

pH – increasing or decreasing the pH of the buffer will change the net charge of the proteins depending on their pI and therefore influence the extent of non-specific ionic interactions.

DTT – this reducing agent is oft en used to prevent loss of enzyme function via oxidation during protein isolation.

EDTA – this chelation agent binds divalent cations and can be used to prevent the action of certain enzymes that require ions such as Mg^{2+} or Ca^{2+}. It can also prevent protein-protein interactions that are dependent on the presence of cations.

Phosphatase inhibitors – when active kinases or phosphorylated proteins are to be isolated, we recommend the addition of 1 mM activated sodium orthovanadate (not compatible with DTT) and 1–10 mM NaF to inhibit phosphatase activity.

We recommend using a fresh tip every time that hot elution buffer is pipetted to ensure reproducible elution volumes are obtained.

4. Troubleshooting

Slow column – if the column begins to run slowly it could be due either to cell debris present in the lysate occluding the column or to air bubble formation within the column. Cell debris should be efficiently removed by high-speed centrifugation (>10,000 g) before addition of the MACSflex MicroBeads. To prevent air bubble formation, use room-temperature buffers for the wash steps or (where possible) degas the buffers before use.

No protein recovery – can be caused by insufficient lysis. The lysis buffer should be altered to optimise the recovery of the tagged target protein (refer to section 2.1 and 3.4). Too stringent lysis or wash buffers may also impair the binding of antibody to the epitope tag, in this case a lower salt concentration may help.

Protein background – if many background protein bands are present following SDS-PAGE analysis of the eluates, a more stringent lysis and/or wash buffer (with higher salt concentrations) should be used (refer to section 3.4).

Background smear – if a smear is seen following SDS-PAGE analysis of the eluates, suitable protease inhibitors should be added to the lysis and wash buffers (refer to 5. Appendix, 5.1).

5. Appendix

5.1 Protease inhibitors

Inhibitor	Final concentration	Stock solution preparation
α1-Antitrypsin (S)	10 µM	6 mg/mL (1000×) in ddH_2O, pH 7
Aprotinin (S)	0.3 µM	1 mg/mL (500×) in ddH_2O, pH 7
Benzamidin (S)	2 mM	3 mg/mL (10×) in ddH_2O, pH 7
EDTA-Na_2 (M)	1 mM	0.5 M (500×) in ddH_2O, pH 8
E-64 (C)	10 µM	0.36 mg/mL (100×) in 1:1 mixture ddH_2O, pH 7: EtOH
Leupeptin (S, C)	10 µM	5 mg/mL (1000×) in ddH_2O, pH 7
PMSF (S)	1 mM	17 mg/mL (100×) in Ethanol, Isopropanol or Methanol. Inactivated by DTT

Cocktails recommended, for example, PMSF, Leupeptin, Aprotinin:

S Serine proteases

M Metalloproteases

C Cysteine proteases

5.2 Preparation of nuclear extracts from cells

Nuclear extraction buffers (NE):

- **Buffer NE-1:** 10 mM HEPES-KOH, pH 7.9, 2.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, Protease inhibitors (e.g. 1 mM PMSF, 10 µM Leupeptin (add protease inhibitors fresh before use)). (Optional) Phosphatase inhibitors (e.g. 10 mM NaF, 1 mM Na_3VO_4).
- **Buffer NE-2:** 20 mM HEPES-KOH, pH 7.9, 2.5 mM $MgCl_2$, 0.42 M NaCl, 25% (v/v) Glycerol, 0.2 mM EDTA, 0.5 mM DTT, Protease inhibitors. (Optional) Phosphatase inhibitors.
- **Buffer NE-3:** 20 mM HEPES-KOH, pH 7.9, 2.5 mM $MgCl_2$, 100 mM KCl, 20% (v/v) Glycerol, 0.2 mM EDTA, 0.5 mM DTT, Protease inhibitors. (Optional) Phosphatase inhibitors.

Preparation of nuclear extracts from activated cells

1. Stimulate approximately 1×10^8 cells to obtain activated transcription factors.
2. Harvest the cells by centrifugation for 10 minutes at 300×g.
3. Wash the cells in PBS (ice cold) and centrifuge again.
4. Place the cell pellet on ice and carry out all further steps on ice.
5. Estimate the volume of the cell pellet and resuspend in 5 volumes of buffer NE-1 by pipetting and incubate for 10 minutes to allow swelling.
6. Centrifuge for 10 minutes at 300×g, 4 °C.
7. Resuspend the cell pellet in 2–3× cell pellet volume of Buffer NE-1 (ice cold).
8. Lyse the cells by douncing in a glass homogenizer and by shearing with a 23G needle.
9. Spin down the nuclei for 10 minutes at 250×g, 4 °C. Discard the supernatant.

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10. Resuspend the pellet in buffer NE-2 (icecold) (1 mL per $0.5–1\times10^8$ cells).

    ▲ **Note:** The addition of 1 M NDSBs (non detergent sulfobetaines, e.g. NDSB-201) to buffer NE-2 may enhance the recovery of nuclear proteins.

11. Resuspend the pellet by sonication. The pellet must be completely solubilized to achieve lysis of the nuclei!

12. Incubate for 30 minutes on ice, rock gently.

13. Centrifuge for 30 minutes at ≥25,000×g, 4 °C. The supernatant contains the nuclear proteins.

14. Dialyze the supernatant against 50× supernatant volume of buffer NE-3 for 5 hours.

6. References

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All protocols and data sheets are available at www.miltenyibiotec.com.

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