



Molecular biology reagents

μ MACS™ FactorFinder Kit User Manual

Kit for the isolation of transcription factors using biotinylated DNA

For 20 reactions

Order No. 130-092-317

Miltenyi Biotec



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The cover photo shows a replica of the DNA model built in 1953 by James D. Watson and Francis Crick at the Cavendish Laboratory in Cambridge.

This model is located at Heureka, the Finnish Science Centre. Photography by Alexander Budde; © Miltenyi Biotec GmbH, Germany. Detailed information on the history of the Watson-Crick model can be found in: de Chadarevian, S. (2003) Relics, replicas and commemorations. Endeavour 27: 75–79.

1. Description

Components	2 mL of μ MACS™ Streptavidin MicroBeads 5 mL Cell Lysis Buffer* 12 mL Binding Buffer 10 mL Wash Buffer LS 10 mL Wash Buffer HS 4 mL Native Elution Buffer* 0.5 mL Binding Enhancer (100× stock solution containing 750 mM $MgCl_2$ and 300 mM $ZnCl_2$)
Size	For 20 reactions. The yield of transcription factors depends on the capture DNA, DNA concentration, and type and size of the protein to be isolated. Typical yields per reaction are in the range from 100 ng to 1 μ g protein.
Product format	μ MACS Streptavidin MicroBeads are supplied in a colloidal suspension containing 0.05% sodium azide.
Storage	Store protected from light at 4–8 °C. Do not freeze. The expiration dates are indicated on the vial labels.

* Cell Lysis Buffer and Native Elution Buffer contain 2 mM EDTA.



1.1 An introduction to transcription factors

The regulation of gene transcription is an expanding research field in which critical steps in the development of cancer and other diseases can be identified. The regulation of gene expression also plays a major role in the process of differentiation during development. Gene expression can be regulated at two levels: at the level of transcription and at the level of translation. Transcriptional control is accomplished by the binding of proteins (transcription factors) to specific regulatory DNA sequences.

Upon binding to specific DNA sequences in the promoter region of a gene, transcription factors (TFs) either activate or repress the transcription of DNA into RNA. Most transcription factors regulate the expression of multiple target genes by forming dimeric or multimeric DNA-binding complexes with other TFs. Transcription factors have a three-domain structure consisting of DNA-binding, activation and protein-protein interaction domains. The possibility of domain interchange between TF families gives rise to enormous transcription factor diversity.

Transcription factors are present in the cytosol and/or nucleus. They are regulated and activated by enzymatic modification, usually by phosphorylation. Upon activation they are transferred from the cytosolic compartment into the nucleus, where they regulate transcription initiation.

1.2 MACS® Technology for the isolation of transcription factors

A DNA oligonucleotide containing the recognition sequence to which a TF binds can be used as a capture molecule to isolate specific TFs. Coupling a biotin moiety to the oligonucleotide allows its specific isolation using streptavidin, a protein which binds biotin with extremely high affinity.

In general, the isolation of a TF protein from eukaryotic cells consists of the following steps: complete lysis of cells (including nucleus), binding of the specific TF to biotin-labeled capture DNA, removal of non-specifically bound proteins, and elution of the TF. It is important to maintain the phosphorylation state of the targeted transcription factors throughout the entire isolation process.

μ MACS™ Streptavidin MicroBeads are superparamagnetic particles coated with streptavidin. They are used to bind the complex formed by biotinylated capture DNA (which contains the specific TF recognition sequence) and the specific TF. Thus, the target proteins are magnetically labeled allowing their specific isolation from the cell lysate.

Following binding of the biotinylated capture DNA to the μ MACS Streptavidin MicroBeads, separation of the DNA-TF complex is achieved by applying a magnetic field: the TF labeled with MicroBeads is retained on MACS® μ Columns by the strong magnetic field of a permanent magnet (the μ MACS or thermoMACS Separator). By simply washing the column with buffer, unbound proteins are effectively removed without affecting the magnetically labeled proteins. Finally, the fraction containing the native transcription factor is eluted while μ MACS Streptavidin MicroBeads and the biotinylated capture DNA remain in the



1. Description

column. Since the MicroBeads are extremely small (approx. 50 nm in diameter), the reaction is fast, and the entire isolation requires less than 30 minutes.

The combination of MACS Column Technology with μ MACS Streptavidin MicroBeads provided in the μ MACS FactorFinder Kit offers a rapid and convenient procedure to isolate high-purity transcription factors (see figure 1).

The kit provides:

- efficient lysis using an optimized buffer,
- fast procedure,
- phosphatase inhibitors, already included in buffers,
- proven MACS Column Technology,
- different wash buffers and a special binding master mixture;
- elution of TF with either high-salt buffer (native conditions) or SDS sample buffer (denaturing conditions).

1.3 Product applications

The μ MACS FactorFinder Kit is well suited for the isolation of DNA-binding transcription factors using eukaryotic cells, prepared cell lysates, or protein solutions as starting material. Using native elution conditions, the isolated TF proteins retain their functionality and can be used directly for assays, such as analysis of DNA sequence motifs with regard to binding specificity or in-column enzymatic reactions. For incubation at 37 °C or 42 °C the temperature-controlled thermoMACS™ Separation Unit is available.

Alternatively, the isolated TF proteins can be further analyzed using methods such as SDS-PAGE, Western blotting, mass spectrometry, etc.



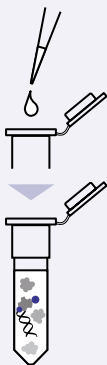
1. Description



1.
Biological sample



2.
Lysis of cells and removal of cell debris



3.
Labeling of target proteins with μ MACS Streptavidin MicroBeads and biotinylated capture probe (e.g. biotinylated DNA)

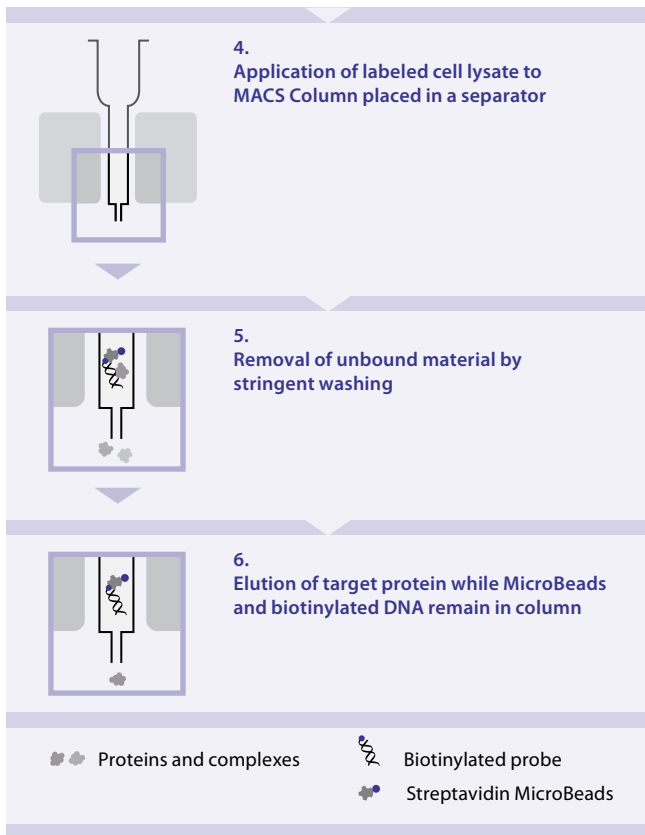


Figure 1: General working scheme for the isolation of transcription factors with biotinylated capture DNA.

1.4 Reagent and instrument requirements

- Biotinylated double-stranded capture DNA, containing the TF-binding site (see 1.5)
- μ Columns (# 130-042-701)
- μ MACS Separation Unit (# 130-042-602) or thermoMACS Separation Unit (# 130-091-136)
- Protease inhibitors, e.g. 1 mM PMSF (prepare 100 mM stock solution in isopropanol and store at room temperature), or 10 μ M leupeptin (prepare 10 mM stock solution in H₂O and store in aliquots at -20 °C)
- Additional phosphatase inhibitors (appropriate buffers already contain phosphatase inhibitors, but Cell Lysis and Binding Buffer can additionally be supplemented, e.g. with 1 mM activated sodium orthovanadate and 50 μ M phenylarsine oxide)
- PBS (1 \times): 137 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, pH 7.2–7.4

- PBS (phosphate buffered saline) containing 5 mM EDTA
- 1.5 mL tubes
- (Optional) buffer for annealing oligonucleotides (see 2.3.1): 10 mM Tris/HCl pH 8.0, 1 mM EDTA, 50 mM NaCl
- (Optional) cofactors, reducing agents or additional reaction components, if required for transcription factor binding to DNA (e.g. Mg^{2+} , Zn^{2+} , BSA, DTT)
- (Optional) poly(dI:dC), e.g. from Sigma-Aldrich (# P4929), for blocking non-specific binding to capture DNA
- (Optional) SDS sample buffer for denaturing elution, e.g. 50 mM Tris/HCl pH 6.8, 50 mM DTT, 2% SDS, 0.01% bromphenol blue, 10% glycerol
- (Optional) blocking reagent for Western blots: 5% non-fat dry milk in TBS / 0.1% Tween[®]-20 (TBS: 150 mM NaCl, 20 mM Tris/HCl, pH 7.5)



1.5 Design of biotinylated capture DNA

In order to bind and to isolate transcription factors, a capture DNA containing the recognition sequence of the transcription factor must be generated. This can be accomplished by direct DNA oligonucleotide synthesis or by PCR amplification.

Oligonucleotide synthesis: Two complementary single-stranded oligonucleotides (HPLC grade) are synthesized. Both should carry a biotin residue at the 5' end. The recognition sequence of the transcription factor should be in the middle of the oligonucleotide sequence with spacers of 10–15 base pairs on both sides (the spacers should not contain any protein binding site sequence). For the binding reaction, the two single-stranded oligonucleotides must be annealed to form a double-stranded capture DNA (see section 2.3). In summary, a functional capture DNA consists of a biotin moiety followed by a spacer of at least 10 base pairs, the recognition sequence, at least 10 additional base pairs and a second biotin.

PCR amplification: This method uses PCR amplification of a 100–200 base pair sequence which contains a promoter and the transcription factor recognition sequence. Both primers (HPLC grade) must carry a 5' biotin. The primers should be designed in such a way that the recognition sequence of the transcription factor is located approximately in the middle of the PCR fragment. The amplified PCR fragment should be purified (by agarose gel extraction and phenol precipitation or equivalent) before it is used for the isolation of transcription factors.

For control purposes, a capture DNA lacking the recognition sequence or containing a scrambled recognition sequence may be useful.

2. General protocol

The μ MACS FactorFinder Kit protocol is optimized for the isolation of transcription factors from mammalian cells. When using plant, yeast or bacterial cells as starting material, the cell lysis procedure needs to be adapted (see Sambrook and Russell (2001) for yeast and bacterial cell lysis procedures, or Dashek (1997) for plant material lysis protocols).

If desired, a positive control experiment can be carried out as described in the Appendix.

2.1 Before starting

▲ Warm all buffers except the Cell Lysis Buffer to room temperature prior use. Put Cell Lysis Buffer on ice.

▲ The volumes of reagents given in this protocol are suitable for processing 10^7 cells. If using less starting material, the same volumes of buffers, oligonucleotide solution, and μ MACS Streptavidin MicroBeads should be used. For isolation of transcription factors expressed at extremely low level, the amount of starting material can be scaled up to approx 2.5×10^7 cells (depending on cell type). In this case, the amount of buffers used should be increased accordingly; however, the volumes of oligonucleotide and MicroBeads solutions specified in this protocol should not be increased.



▲ In order to reduce potential non-specific binding of proteins to the capture DNA, a final concentration of 50 ng/mL poly(dI:dC) can be added to Binding Buffer, Wash Buffer LS and Wash Buffer HS.

▲ If it is not known whether the binding of the transcription factor to DNA requires metal ions, addition of 1× Binding Enhancer (final concentration) to the Binding Buffer is recommended.

▲ Protease inhibitors and additional phosphatase inhibitors (optional) should be added to ice-cold Cell Lysis Buffer immediately before use (see protocol step 3 in 2.2).

▲ For optimal control of isolation, it is recommended that all wash and elution fractions are collected separately for SDS-PAGE and Western blot analysis. For SDS-PAGE, transcription factors can also be eluted with hot SDS sample buffer.

2.2 Preparation of cell lysates

▲ Perform cell lysis on ice.

▲ In general, cells must be stimulated for the isolation of activated transcription factors. Since activated transcription factors are phosphorylated, the appropriate kit buffers already contain phosphatase inhibitors. If desired, additional phosphatase inhibitors may be added to the Cell Lysis and Binding Buffers. .

1. Collect 10^7 cells by centrifugation for 5 min at $300\times g$ and $4\text{ }^{\circ}\text{C}$.

2. Wash cells twice: add 1 mL PBS containing 5 mM EDTA, centrifuge at 300×g for 5 min at 4 °C.
3. Add protease inhibitors and phosphatase inhibitors (optional) to the required amount of Cell Lysis Buffer (stored on ice), e.g. add 1 μ L of 100× protease inhibitor stock solution and 1 μ L of 100× phosphatase inhibitor stock solution to 100 μ L Cell Lysis Buffer per 10^7 cells.
4. Resuspend cells in 100 μ L ice-cold Cell Lysis Buffer.
5. Lyse cells by three freeze-and-thaw cycles: Place cells at 37 °C for 2 min followed by -70 °C for 5 min. Repeat twice.
▲ Note: The freeze step at -70 °C can be replaced by placing cells in liquid nitrogen for a short time.
6. Centrifuge the lysate for 5 min at 15,000×g and 4 °C.
7. (Optional) store supernatant (= cleared cell lysate) at -70 °C.
▲ Note: For control purposes, the pellet should also be stored at -70 °C.



2.3 Generation of capture DNA

Use protocol 2.3.1 or 2.3.2 to generate biotinylated capture DNA. See also 1.5 “Design of biotinylated capture DNA”.

2.3.1 Annealing of oligonucleotides to form capture DNA

1. Denature 0.75 μg of each single-stranded oligonucleotide (= 1.5 μg total DNA) per capture assay in 10 mM Tris/HCl, pH 8, 1 mM EDTA, 50 mM NaCl for 3 min at 95 °C.
2. Allow the solution to cool down slowly to room temperature to anneal the oligonucleotides. For convenience, a thermal cycler with a cooling program of e.g. 95 °C \rightarrow 25 °C in 60 min can be used.
▲ Note: It is important that the capture DNA is in a double-stranded form; this can be checked by polyacrylamide gel electrophoresis. If many single-stranded molecules are present, high background binding may be seen due to the presence of abundant proteins that bind single-stranded nucleic acids.
3. Store at -20 °C. Avoid multiple freeze-thaw cycles.

2.3.2 PCR generation of capture DNA

1. Perform PCR with 5' biotinylated primers to amplify the desired DNA fragment.
2. Purify biotinylated DNA fragment (see Sambrook and Russell 2001).
3. Store at -20 °C. Avoid multiple freeze-thaw cycles.

2.4 Magnetic isolation of transcription factors

▲ It is recommended that all wash and elution fractions are collected separately for SDS-PAGE and Western blot analysis (optional).

1. Place a μ Column in the μ MACS or thermoMACS Separator.
2. Mix 1 volume of cell lysate with 2 volumes of Binding Buffer. Add protease inhibitors, phosphatase inhibitors, and Binding Enhancer (optional) to Binding Buffer before mixing. Add 50 pmol biotinylated capture DNA.

Example: For 100 μ L cell lysate, add 200 μ L Binding Buffer and 1.5 μ g double-stranded biotinylated DNA (40 bp) or 5 μ g double-stranded biotinylated DNA (150 bp).

▲ **Note:** The required amount of cell lysate must be determined experimentally due to the different abundance of various transcription factors.

▲ **Note:** If the binding of the transcription factor to DNA depends on additional reaction components such as cofactors or reducing agents, the Binding Buffer must be supplemented (e.g. to a final concentration of 7.5 mM Mg^{2+} , 3 mM Zn^{2+} , 0.1–1 mM BSA, 1–5 mM DTT). If details are not known, add 1 μ L Binding Enhancer (100 \times stock solution) per 100 μ L Binding Buffer.

3. Incubate for 20 min at room temperature.

▲ **Note:** Depending on the transcription factor, the incubation time may be varied. It is important that the binding of the transcription factor to the capture DNA reaches equilibrium. This incubation time must be determined experimentally. If the incubation is carried out on ice, longer incubation times may be required.



2. General protocol

4. Add 100 μL μMACS Streptavidin MicroBeads and mix briefly.
5. Incubate for 10 min at room temperature.
6. Apply 100 μL Binding Buffer onto μ Column to rinse.
7. Apply reaction mixture onto the column matrix. Allow it to flow through (optional: collect flow-through).
8. Apply 4 times 100 μL Wash Buffer LS (low stringency) sequentially onto μ Column (optional: collect wash fractions). Each time allow buffer to run through.
9. Apply 4 times 100 μL Wash Buffer HS (high stringency) sequentially onto μ Column to wash (optional: collect wash fractions). Each time allow buffer to run through.
 - ▲ **Note:** Depending on the strength of the binding interaction, the transcription factor may already elute at this step.
10. For the elution, add 30 μL Native Elution Buffer onto the μ Column. Discard the flow-through and wait for 2 min.
 - ▲ **Note:** Addition of 0.5 mg/mL BSA to Elution Buffer may stabilize transcription factors.
 - ▲ **Note:** For SDS-PAGE, transcription factors can also be eluted with 50–100 μL 95 °C hot SDS sample buffer.
11. Elute with 50 μL Native Elution Buffer.
 - ▲ **Note:** For a higher yield but less concentrated eluate, add an additional 50 μL Elution Buffer and collect flow-through. This results in about 30% more transcription factor in a total volume of 100 μL .

3. Troubleshooting

With the μ MACS FactorFinder Kit, highly pure transcription factors can be isolated. However, if problems are encountered, the following section will help to identify the cause and provide a solution.

Yield of isolated transcription factor is low or zero

Check whether transcription factor is present in cell lysate, flow-through, and/or wash fractions.

Low amounts of transcription factor in cell lysate

Insufficient stimulation of cells:

check for appropriate cell stimulation (e.g. by cytokines) to obtain high amounts of phosphorylated transcription factor.

Inefficient cell lysis:

lyse cells by three freeze-and-thaw cycles in ice-cold Cell Lysis Buffer to disrupt cells and nuclei (see protocol); do not use less than 100 μ L Cell Lysis Buffer per 10^7 cells.

Sample material (e.g. plant, yeast, bacteria) unsuitable for protocol:

kit protocol can only be used for efficient lysis of eukaryotic cells without cell walls.



Transcription factor proteolytically degraded:
add freshly prepared protease inhibitors to Cell Lysis Buffer and Binding Buffer (see 2.2, step 3); work on ice if possible.

Transcription factor dephosphorylated:
supplement Cell Lysis Buffer, Binding Buffer, and both Wash Buffers with additional phosphatase inhibitors (see 2.2, step 3); work on ice if possible.

Transcription factor mainly in flow-through fraction

Insufficient stimulation of cells:
check for appropriate cell stimulation (e.g. by cytokines) to obtain high amounts of phosphorylated transcription factor.

Suboptimal capture DNA sequence:
check if there is a more effective transcription factor binding site to be used as capture DNA.

Spacer sequence on capture DNA too short:
use spacers of at least 10–15 base pairs between capture sequence and 5' biotin moieties.

Biotin absent from capture DNA:
use primers or oligonucleotides with 5' biotin moieties.

Low quality of capture DNA:
use HPLC-purified PCR primers or oligonucleotides. Clean up biotinylated PCR products before use.

Insufficient annealing of oligonucleotides for capture DNA:
anneal oligonucleotides slowly (see 2.3.1) and check by PAGE.

Too little capture DNA:

increase amount of capture DNA between 2–4 fold.

Too much capture DNA:

decrease amount of capture DNA between 2–4 fold.

Missing cofactors for binding reaction (metal ions etc.):

add necessary cofactors to Binding Buffer (if known); add Binding Enhancer to Binding Buffer (i.e. 1 μ L Binding Enhancer [100 \times] per 100 μ L Binding Buffer).

Incubation time for binding reaction of transcription factor to capture DNA too short:

extend incubation time (see 2.4, step 3).

Transcription factor dephosphorylated:

supplement Cell Lysis Buffer, Binding Buffer, and both Wash Buffers with additional phosphatase inhibitors (see 2.2, step 3); work on ice if possible.

Abnormally high amounts of free biotin present in cell lysate:

remove free biotin, e.g. with μ MACS Streptavidin MicroBeads, before incubating cell lysate with capture DNA and μ MACS Streptavidin MicroBeads.



Abnormally high amounts of free biotin present in biotinylated capture DNA:

use HPLC grade primers/oligonucleotides.

Transcription factor mainly in wash fractions

Weak binding of transcription factor to capture DNA:

check if there is a more effective transcription factor binding site for the capture DNA.

High amounts of transcription factor in cell lysate, but low amounts in flow-through, wash, and elution fractions

Very strong binding of transcription factor to capture DNA:

- native conditions: add 10 μL of 5 M NaCl to 90 μL of Native Elution Buffer.
- denaturing conditions: elute with hot SDS sample buffer.
- increase incubation time after addition of 30 μL Native Elution Buffer from 2 min up to 5 min.

Western blot-related problems

Incorrect antibody concentrations:

titrate concentrations of the primary and secondary antibodies.

Transcription factor not detected by phospho-specific antibody:

check that the antibody detects the specific phosphorylation that results in transcription factor binding to DNA.

Weak signal:
use chemiluminescent detection method.

High background of other proteins in transcription factor fraction

Too much debris in cell lysate:
centrifuge lysate at $\geq 15,000\times g$ for 5 min.

High amounts of DNA-binding proteins in cell lysate:
add poly(dI:dC) to Binding Buffer, Wash Buffer LS, and Wash Buffer HS.

Other proteins bind to spacer sequence of capture DNA:
use a spacer sequence without binding motifs for other DNA-binding proteins.

Serum and/or albumin in cell growth medium:
use serum-free medium, or wash cell pellet extensively.

Too much cell lysate used:
reduce the amount of cell lysate.

Suboptimal capture DNA:
check if there is a more effective transcription factor binding site for the capture DNA.

Insufficient annealing of oligonucleotides for capture DNA:
anneal oligonucleotides slowly (see 2.3.1) and check by PAGE.



Insufficient washing steps on μ Column:

stepwise increase NaCl concentration: add 4 μ L of 5 M NaCl to 400 μ L Wash Buffer HS and wash with 4 \times 100 μ L Wash Buffer (first additional wash step). Add 8 μ L of 5 M NaCl to 400 μ L Wash Buffer HS and wash with 4 \times 100 μ L Wash Buffer (second additional wash step).

Western blot-related problems

Blot insufficiently blocked:

block according to antibody manufacturer's recommendation or block with 5% non-fat dry milk in TBS / 0.1% Tween[®]-20 for 1 hour at room temperature.

Blot insufficiently washed:

wash according to antibody manufacturer's recommendation or 3 times with TBS / 0.1% Tween-20.

Incorrect antibody concentrations:

titrate concentrations of the primary and secondary antibodies.

Cross-reactivity of polyclonal antibody:

use monoclonal antibody.

Buffer either does not flow or flows very slowly through μ Column

Too much debris in cell lysate:

Centrifuge lysate at $\geq 15,000\times g$ for 5 min.



4. References

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5. Appendix: Control experiment

The control experiment can be used as a positive control and to implement the procedure. In this experiment STAT3 (signal transducer and activator of transcription 3) is purified using a capture DNA constructed from the following two 5'-biotinylated oligonucleotides:

5' — GCT AGA CAT CTC GAG GAT CCT TCT GGG AAT TCC TAG
ATC — 3' (STAT3 oligo 1)

5' — GAT CTA GGA ATT CCC AGA AGG ATC CTC GAG ATG TCT
AGC — 3' (STAT3 oligo 2)

The two oligonucleotides are annealed according to the protocol in section 2.3.

5.1 Preparation of activated T cell lysate

For the isolation of STAT3 from T cells, 50–100 μL activated T cell lysate per assay is required.

5.1.1 Reagent and instrument requirements

- Medium (X-VIVO 15™ (Cambrex) supplemented with 5% human AB serum)
- PE buffer (PBS containing 5 mM EDTA and 0.5% human AB serum)
- MACSmix™ tube rotator (# 130-090-753)



- MACSiMAG™ Separator (# 130-092-168)
- T Cell Activation/Expansion Kit (# 130-091-441)
- 6-well plate flat-bottom cell culture plate with lid

5.1.2 Protocol

5.1.2.1 Before starting

To prepare the PE buffer, inactivate human AB serum prior use by incubating at 56 °C for 30 min.

Load Anti-Biotin MACSiBead™ particles according to the protocol in the T Cell Activation/Expansion Kit data sheet.

5.1.2.2 Preparation of loaded Anti-Biotin MACSiBead™ particles and mononuclear cells from peripheral blood

1. Wash 1×10^8 loaded Anti-Biotin MACSiBead particles in 5 mL medium.
2. Centrifuge at 300×g for 5 min.
3. Repeat steps 1–2.
4. Resuspend loaded Anti-Biotin MACSiBead particles in 4 mL medium.
5. Wash 2×10^8 PBMCs in 5 mL medium.
6. Centrifuge at 300×g for 5 min.
7. Repeat steps 5–6.

8. Resuspend cells in 4 mL medium.

5.1.2.3 T cell activation from peripheral blood mononuclear cells

To stimulate 2×10^8 PBMCs with 1×10^8 loaded Anti-Biotin MACSiBead particles follow the below mentioned steps based upon the T Cell Activation/Expansion Kit protocol (using one loaded Anti-Biotin MACSiBead particle per two cells).

1. Plate 1 mL cell suspension (5×10^7 cells) per well and add 1 mL (2.5×10^7) loaded Anti-Biotin MACSiBead particles in a 6-well flat-bottom cell culture plate with lid, and mix.
2. Add 8 mL medium per well (total volume: 10 mL per well) and incubate at 37°C und $7.5\% \text{CO}_2$ for three days.
▲ Note: Inspect cultures daily, and add fresh medium if required.
3. Stimulate T cells by adding 10 ng IL-2 per mL culture (corresponding to 100 ng IL-2 per well).
4. Incubate at 37°C and $7.5\% \text{CO}_2$ for three days.
5. Collect cells by carefully rinsing the cell culture plate with a pipette and pool cells (4 wells, 2×10^8 cells) into a 50 mL tube. Do not rinse too vigorously as this results in detachment of contaminating monocytes.
6. Centrifuge at $300 \times g$ for 5 min.
7. Discard supernatant.



8. Resuspend cells in 1 mL PE buffer. Transfer to 15 mL conical tube and add 9 mL PE buffer.
9. Centrifuge at 300×g for 5 min.
10. Discard supernatant.
11. Resuspend cells in 5 mL PE buffer.

5.1.2.4 Removal of Anti-Biotin MACSiBead™ particles

Separate cells from Anti-Biotin MACSiBead™ particles using the MACSiMAG™ Separator.

1. Place the tube in the magnetic field of the MACSiMAG Separator.
2. Allow the MACSiBead particles to adhere to the wall of the tube for 2 min.
3. Retaining the tube in the magnet, carefully remove the supernatant containing the MACSiBead-depleted cells. Collect supernatant in a new 15 mL tube.
4. Remove the tube from the separator and add 5 mL PE buffer.
5. Vortex sample, replace tube in the MACSiMAG Separator and wait for 2 min.
6. Collect entire supernatant in the same tube as used in step 3; total volume should be 10 mL.
7. Centrifuge at 300×g for 5 min.
8. Discard supernatant.

9. Resuspend cell pellet with 1 mL medium.
10. Add 19 mL medium.

5.2 Activation of STAT3

1. Transfer cells into 6-well flat-bottom cell culture plate with lid (10 mL per well).
2. Incubate at 37 °C und 7.5% CO₂ overnight.
3. Split content of each well into 2 new wells.
4. For the activation of STAT3, stimulate cells of one well with IL-15: Add 50 ng IL-15 per mL culture (corresponding to 250 ng IL-15 per well). Alternatively, IL-2 can be used. The other well serves as an unstimulated control.
5. Incubate at 37 °C for 15 min.

Now start working on ice and use ice-cold PE buffer and Cell Lysis buffer!

6. Transfer cells from well to 15 mL tube. Use a scraper to collect all cells.
7. Rinse wells with PE buffer and transfer to tube containing cells. Fill up to a total volume of 15 mL with PE buffer.
8. Continue with protocol, step 2.2.1. Use 50–100 μ L of the lysate, 1.5 μ g of the annealed STAT3 capture DNA and 100–200 μ L of Binding Buffer.



5.3 Western blot

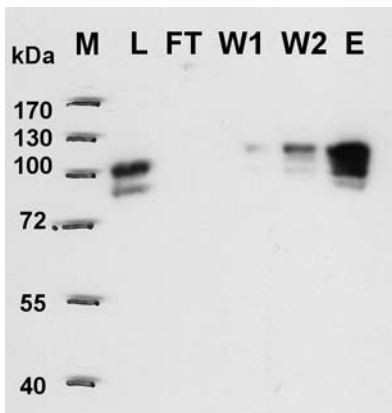
STAT3 isolation can be analyzed with a Western blot.

1. Use 10–20 μL cell lysates (unstimulated and stimulated with IL-15; these serve as control for the activation/phosphorylation of STAT3 in the T cells) and as much as possible of the collected fractions. The volumes can be reduced by using a SpeedVac.
2. Block membrane with TBS / 0.1% Tween-20 / 5% non-fat dry milk at room temperature for 1 hour.
3. Add Phospo-Stat3 (Tyr705, 3E2) from Cell Signaling Technology (# 9138), mouse monoclonal, 1:1,000 in TBS / 0.1% Tween-20 / 5% non-fat dry milk and incubate at 4 °C overnight.
4. Add goat anti-mouse IgG-HRPO from Dianova (# 115-035-062), 1:10,000 in TBS / 0.1% Tween-20 / 5% non-fat dry milk and incubate at room temperature for 1 hour.
5. Detect signals by chemiluminescence.

5.4 Example of a control experiment

Figure 2 shows a typical result: STAT3 is eluted in the eluate fraction and is nearly absent from the flow-through and wash fractions. The phosphorylated protein was identified on the Western blot using a monoclonal antibody Phospho-STAT3 (Tyr705, 3E2) that specifically recognizes a phosphorylated tyrosine residue at position 705 in the STAT3 amino acid sequence.

Figure 2: Isolation of STAT3 protein as a control experiment. Western blot using a 1:1,000 dilution of the anti-phospho-STAT3 (Tyr705) antibody. Lane M: molecular weight marker; lane L: total, stimulated T-cell lysate (5 μ L); lane FT: flow-through; lanes W1: low-salt wash fractions; lane W2: high-salt wash fraction; lane E: eluate



Warnings

Reagents contain sodium azide. Under acidic conditions sodium azide yields hydrazoic acid, which is extremely toxic. Azide compounds should be diluted with water before discarding. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

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