NanoTag Biotechnologies

Immunoprecipitation Protocol

Immunoprecipitation of fusion proteins from cell extracts using NanoTag's ALFA System

The ALFA-tag is a novel, highly versatile epitope tag (core sequence SRLEEELRRRLTE) that can be employed for a wide range of life science applications (see Götzke 2019[1]). For immunoprecipitation of ALFA-tagged target proteins, NanoTag Biotechnologies offers two types of nanobody-based ALFA Selector resins.

- **ALFA SelectorST** (for ***Super-Tight***) was optimized to reach the highest possible binding strength. It features a nanobody binding ALFA-tagged targets with an affinity of ~26 pM. Elution of target proteins from ALFA SelectorST requires acidic or denaturing conditions.
- **ALFA Selector^{PE}** (for ***Peptide Elution***) displays a nanobody with lower affinity for ALFA-tagged targets (Kd ~11 nM), which was optimized for competitive peptide elution under physiological conditions.
- **ALFA Selector^{CE}** (for ***Cold Elution***) displays a nanobody with lower affinity for ALFA-tagged targets (Kd ~100 nM), which was optimized for competitive elution using physiological buffers at low temperature (i.e. 4°C)

All ALFA Selectors are based on cross-linked 4% agarose beads. The site-directed and chemically stable immobilization of nanobodies allows for a high capacity (>150 µM target protein, e.g., 4.5 mg GFP-ALFA per mL of resin) and optimal accessibility of the available binding sites. At the same time, minimal leakage of nanobodies is ensured also under harsh denaturing and/or reducing conditions. In contrast to conventional immunoprecipitations, the eluates of both ALFA selectors will not contain large amounts of co-eluted antibody fragments. Due to the combination of high capacity, minimal leakage and extraordinary low non-specific protein adsorption, both ALFA Selector resins allow for clean and highly specific immunoprecipitations.

Selector Resins are compatible not only with physiological buffers but also with high stringency buffers (see product-specific compatibility charts) and reducing agents.

Only for research applications, not for diagnostic or therapeutic use!

Products		
ALFA Selector^{PE} **incl. ALFA elution peptide**	2000 µL 50% slurry + 10 mg ALFA elution peptide	Order no. N1510
ALFA SelectorST	2000 µL 50% slurry	Order no. N1511
ALFA Selector^{CE} **incl. ALFA elution peptide**	2000 µL 50% slurry + 10 mg ALFA elution peptide	Order no. N1512

Materials (not included):

• Lysis buffer[1] • Washing buffer[1] • Tris-buffered saline (TBS) pH 7.4 • 2x SDS sample buffer
• Glycine/HCl elution buffer pH 2.2

Remarks

[1] Selector resins are compatible with most common Lysis/Washing buffers (e.g. RIPA). For custom buffers please refer to the product-specific compatibility chart.

For further information concerning this protocol please contact us at
info@nano-tag.com

[1] Götzke H, Kilisch M, Martínez-Carranza M, Sograte-Idrissi S, Rajavel A, Schlichthaerle T, Engels N, Jungmann R, Stenmark P, Opazo F, Frey S. **The ALFA-tag is a highly versatile tool for nanobody-based bioscience applications.** Nat Commun. 2019 Sep 27;10(1):4403.

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Immunoprecipitation Protocol

Protocol using Mini Spin Columns (analytical scale)

We recommend using Mini Spin Columns (Cat. No. A1001) for immunoprecipitation experiments

1. Prepare cell lysates (0.2 to 1.5 ml volume) according to established protocols.
   For mammalian cells, we recommend using 10^6-10^8 cells per experiment.

2. Clear lysate by centrifugation for 10 min at > 14000 x g and 4 °C.
   Take sample for further analysis (Input fraction)

3. Equilibrate ALFA Selector Resin
   a. Resuspend ALFA Selector Resin.
   b. Transfer 20 µl slurry (10 µl packed beads) into a clean 1.5 ml reaction tube.
   c. Add 1 ml lysis buffer.
   d. Centrifuge for 1 min at 1000 x g and carefully remove supernatant.
   e. Repeat steps 3c-3d once.

4. Add cleared lysate from step 2 to equilibrated ALFA Selector resin from step 3.

5. Incubate 1 h at 4 °C with head-over-tail rotation.

6. Sediment beads by centrifugation for 1 min at 1000 x g and 4 °C.
   Take sample from supernatant for further analysis (Non-bound fraction)

7. Washing
   a. Carefully remove supernatant.
   b. Resuspend beads in 1 ml Lysis buffer
   c. Centrifuge for 1 min at 1000 x g.
   d. Remove Supernatant
   e. Repeat steps 7b-7d twice.

8. Transfer
   a. Remove bottom plug from Mini Spin Column. Place column in 2 ml reaction tube.
   b. Resuspend beads in 200 µl Lysis buffer and transfer suspension to Mini Spin Column.
   c. Wash out remaining beads sticking to the tube with 200 µl Lysis buffer,
      Transfer the suspension to the Mini Spin Column.
   d. Centrifuge for 1 min at 1000 x g and discard flow-through.

9. Washing
   a. Add 400 µl Washing buffer
   b. Centrifuge for 1 min at 1000 x g and discard flow-through
   c. Repeat steps 9a-9b once.

10. Wash once with 400 µl TBS, centrifuge for 1 min at 1000 x g.

11. Attach bottom plug and place Mini Spin Column in a clean 1.5 ml reaction tube

12. Elution

Peptide elution under native conditions (ALFA Selector^{PE} and ALFA Selector^{CE})

This elution mode is based on competition between the ALFA elution peptide present in the elution buffer and the ALFA-tagged target protein for available binding sites on the resin. To obtain convenient elution kinetics, peptide elution has to be performed at room temperature (22-25°C) using ALFA Selector^{PE} or at low temperatures (4°C) using ALFA Selector^{CE}.

Acidic elution (ALFA Selector^{PE} / ALFA SelectorST / ALFA Selector^{CE})

ALFA-tagged target proteins can efficiently be eluted from both ALFA Selector variants at low pH. As an Acidic Elution buffer we recommend 0.1 M Glycin/HCl pH 2.2, 150mM NaCl.

Denaturing elution using SDS sample buffer (ALFA Selector^{PE} / ALFA SelectorST)

ALFA-tagged target proteins can efficiently be eluted from both ALFA Selector variants using SDS sample buffer at elevated temperatures.

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Elution procedures:

a. **Peptide elution** (ALFA Selector^{PE} / ALFA Selector^{CE})

i. Prepare a 100x (20 mM) stock solution of ALFA elution peptide by solubilizing the ALFA elution peptide at 40mg/mL in deionized water.
ii. Dilute stock solution 1:100 in physiological buffer to obtain an Elution buffer containing 200 µM ALFA elution peptide
iii. Add 5 column bed volumes (CBV) Elution buffer to the resin.
iv. Incubate for 20 min at room temperature with subtle shaking.
v. Remove the bottom plug and collect the eluate by centrifugation 1 min at 1000 x g.
vi. **Optional:** For highest yields attach bottom plug and repeat steps iii-v. Combine eluates.

b. **Elution under acidic conditions** (ALFA Selector^{PE} / ALFA SelectorST / ALFA Selector^{CE})

i. Prepare Acidic Elution buffer (0.1 M Glycin/HCl pH 2.2, 150mM NaCl)
ii. Add 2.5 column bed volumes (CBV) Acidic Elution Buffer to the resin.
iii. Incubate for 2 min at RT with subtle shaking.
iv. Remove the bottom plug and collect the eluate by centrifugation 1 min at 1000 x g.
v. Attach bottom plug and repeat steps ii-iv once
vi. Combine eluates.

c. **Denaturing elution using SDS sample buffer** (ALFA Selector^{PE} / ALFA SelectorST / ALFA Selector^{CE})

i. Add 2.5 column bed volumes (CBV) SDS sample buffer pre-warmed to ~60°C to the resin.
ii. Close Mini Spin Column with lid.
iii. Incubate for 5 min at 80-90°C with subtle shaking.
iv. Open lid and remove the bottom plug and collect the eluate by centrifugation 1 min at 1000 x g.
v. Attach bottom plug and repeat steps ii-iv once
vi. Combine eluates.

13. Take sample from elution for further analysis (elution fraction)

14. Analyze collected samples by SDS-PAGE.

NanoTag Biotechnologies

Immunoprecipitation Protocol

Protocol for preparative purifications using gravity flow

1. Prepare native cell lysates according to established protocols.

2. Clear lysate by centrifugation for 10 min at > 14000 x g and 4 °C.

3. Equilibrate ALFA Selector Resin^{PE} or Resin^{CE}
   a. Resuspend ALFA Selector Resin.
   b. Transfer slurry to a clean gravity flow column
   c. Wash resin with 5 column bed volumes (CBV) of Lysis buffer.

4. Binding

a. Binding in batch mode

i. Add equilibrated ALFA Selector Resin (step 3) to the cleared lysate (step 2).
ii. Incubate 1 h at 4 °C with rotation.
iii. Sediment beads by centrifugation for 1 min at 1000 x g and 4 °C.
iv. Discard the supernatant and transfer beads into the empty gravity flow column used in step 3.

b. Binding in column mode

i. Slowly pass cleared lysate obtained in step 2 over the equilibrated column, collect flow-through.
ii. Optional if required: Pass non-bound material (flow-through) once more over the column.

5. Wash resin with 10-20 CBV Washing buffer

6. Elution

Peptide elution under native conditions (ALFA Selector^{PE} / ALFA Selector^{CE})

This elution mode is based on competition between the ALFA elution peptide present in the elution buffer and the ALFA-tagged target protein for available binding sites on the resin. To obtain convenient elution kinetics, peptide elution has to be performed at room temperature (22-25°C) using ALFA Selector^{PE} or at low temperatures (4°C) using ALFA Selector^{CE}.

Acidic elution (ALFA Selector^{PE} / ALFA SelectorST / ALFA Selector^{CE})

ALFA-tagged target proteins can efficiently be eluted from both ALFA Selector variants at low pH. As an Acidic Elution buffer we recommend 0.1 M Glycin/HCl pH 2.2, 150mM NaCl.

a. Competitive elution with ALFA elution peptide (ALFA Selector^{PE} / ALFA Selector^{CE})

Note: We recommend performing peptide elution at room temperature (RT; 22-25°C) for ALFA Selector^{PE}. At at 4°C we recommend ALFA Selector^{CE} .

i. Prepare 500 µM (0.9 mg/mL) ALFA elution peptide in native buffer.
ii. Add 1.2 CBV elution buffer (equilibrated to RT) prepared in step i on top of the resin.
iii. Collect eluate fraction and incubate 10-15 min at RT.
iv. Repeat steps ii-iii 2-3 times.
v. Analyze eluate fractions for content of target protein.

b. Elution under acidic conditions
(ALFA Selector^{PE} / ALFA SelectorST / ALFA Selector^{CE})

Note: Please make sure that the target protein is compatible with acidic elution conditions. Acidic elution can be performed at room temperature or at 4°C.

i. Prepare Acidic Elution buffer (0.1 M Glycin/HCl pH 2.2, 150mM NaCl)
ii. Add 1.2 CBV elution buffer prepared in step i on top of the resin.
iii. Collect eluate fraction and immediately neutralize by adding 1/10 volume 1M Tris pH 8.5
iv. Incubate 1-2 min.
v. Repeat steps ii-iv 2-3 times.
vi. Analyze eluate fractions for content of target protein.

7. Analyze eluate fractions for content of target protein.

8. Analyze input material, non-bound material and eluate fractions by SDS-PAGE.

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