NanoTag Biotechnologies

ALFA Selector Resins

Immunoprecipitation of fusion proteins from cell extracts using ALFA Selector Resins

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[1]. For immunoprecipitation of ALFA-tagged target proteins, NanoTag Biotechnologies offers ALFA Selector Resins[1,2] based on different nanobody variants.

- **ALFA SelectorST** (for ***Super Tight***) is designed to reach the highest possible binding strength. It features a nanobody binding ALFA-tagged targets with an affinity of ~26 pM[1]. Efficient 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) and is optimized to allow for competitive peptide elution under physiological conditions[1].
- **ALFA Selector^{CE}** (for ***Cold Elution***) displays a nanobody with lower affinity for ALFA-tagged targets (Kd ~100 nM). The resin is ideal for competitive elution using physiological buffers at low temperature (i.e. 4°C) and can also be used for flow elution at room temperature[2].

All ALFA Selector variants are available based on **non-magnetic** or **magnetic 4% agarose beads**. The site-directed and chemically stable immobilization of nanobodies ensures 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 observed 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, all ALFA Selector Resins allow for clean and highly specific immunoprecipitations.

NanoTag's ALFA Selector Resins are widely compatible not only with physiological buffers and most common Lysis/Washing buffers, but also with high stringency buffers, detergents and reducing agents (see compatibility chart on page 8). The resins can therefore be used for customized applications using a wide variety of buffer compositions and pH values.

Relevant products

ALFA SelectorST	4% crosslinked agarose	Cat. No. N1511
2000 µl 50% slurry	4% magnetic agarose	Cat. No. N1516
ALFA Selector^{PE}		
2000 µl 50% slurry	4% crosslinked agarose	Cat. No. N1510
including 10 mg ALFA elution peptide	4% magnetic agarose	Cat. No. N1515
ALFA Selector^{CE}		
2000 µl 50% slurry	4% crosslinked agarose	Cat. No. N1512
including 10 mg ALFA elution peptide	4% magnetic agarose	Cat. No. N1517
ALFA elution peptide	10 mg ALFA elution peptide (lyophilized)	Cat. No. N1520

Materials (not included):

- Buffers: Lysis buffer • Washing buffer • Elution buffer • Tris-buffered saline (TBS) pH 7.4
- 2x SDS sample buffer
- Columns and tubes
- Optional: Magnetic rack

I. Analytical scale purifications using non-magnetic ALFA Selector Resins

We recommend using Mini Spin Columns (Cat. No. A1001) for analytical scale immunoprecipitation experiments using non-magnetic ALFA Selector Resins

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 the 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 Lysis 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 from ALFA Selector^{PE} has to be performed at room temperature (22-25°C). ALFA Selector^{CE} can efficiently be eluted at temperatures between 4 and 25°C. An enhanced elution kinetics is observed at even higher temperatures (e.g. 37°C).

Acidic elution (all ALFA Selector Resins).

ALFA-tagged target proteins can efficiently be eluted from all ALFA Selector variants at low pH. As a general Acidic Elution Buffer, we recommend 0.1 M Glycine/HCl pH 2.2, 150 mM NaCl.

Denaturing elution using SDS sample buffer (all ALFA Selector Resins).

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

I. Analytical scale purifications using non-magnetic ALFA Selector Resins (continued)

Elution procedures:

a. **Peptide elution batch mode** (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 40 mg/ml in deionized water.
ii. Dilute the ALFA peptide stock solution 1:100 in physiological buffer to obtain an Elution buffer containing 200 µM ALFA elution peptide.
iii. Attach bottom plug on column. Add 5 resin volumes (RV) of Elution buffer to the resin.
iv. Incubate with subtle shaking:
ALFA Selector^{PE}: 15-20 min at room temperature.
ALFA Selector^{CE}: 3-5 min at room temperature.
or 15-20 min at 4°C.
v. Remove the bottom plug and collect the eluate by centrifugation for 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** (all ALFA Selector variants).

Please check the general remarks related to acidic elution approaches on page 8.

i. Prepare Acidic Elution Buffer (e.g. 0.1 M Glycine/HCl pH 2.2, 150mM NaCl).
ii. Add 2.5 resin volumes (RV) of 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.
vii. Neutralize eluates by adding e.g. 1/10 volume of 1 M Tris-HCl pH 8.5.

c. **Denaturing elution using SDS sample buffer** (all ALFA Selector variants).

Denaturing elution will in general not be compatible with protein functionality. It is therefore explicitly recommended for analytical applications only.

i. Add 2.5 resin volumes (RV) of SDS sample buffer pre-warmed to ~60°C to the resin.
ii. Close the Mini Spin Column by attaching the lid and bottom plug.
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 fractions for further analysis (Elution fraction).

14. Analyze collected samples by SDS-PAGE.

II. Analytical scale purifications using magnetic ALFA Selector Resins

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 magnetic 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. Collect beads by placing the tube in a magnetic rack and remove supernatant.
   e. Repeat steps 3c-3d once.

4. Add cleared lysate from step 2 to the equilibrated magnetic ALFA Selector Resin from step 3.

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

6. Collect beads by placing the tube in a magnetic rack.
   Take sample from supernatant for further analysis (Non-bound fraction).

7. Washing:
   a. Carefully remove supernatant.
   b. Resuspend beads in 1 ml Lysis buffer and incubate 1-2 min head-over-tail.
   c. Collect beads by placing the tube in a magnetic rack.
   d. Remove supernatant.
   e. Repeat steps 7b-7d three times.
   f. Resuspend beads in 1 ml Lysis buffer and transfer suspension into fresh 1.5 ml tube.
   Note: This step is essential as generally a lot of contaminant proteins stick to the walls of the tube used during the binding step!
   g. Wash beads twice as described in steps 7b-7d.
   h. Wash once with 1 ml TBS and collect beads on magnetic rack.
   i. Remove the supernatant completely.

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 from ALFA Selector^{PE} has to be performed at room temperature (22-25°C). ALFA Selector^{CE} can efficiently be eluted at temperatures between 4 and 25°C. Enhanced elution kinetics are observed at even higher temperatures (e.g. 37°C).

Acidic elution (all ALFA Selector Resins).

ALFA-tagged target proteins can efficiently be eluted from all ALFA Selector variants at low pH. As a general Acidic Elution Buffer we recommend 0.1 M Glycine/HCl pH 2.2, 150 mM NaCl.

Denaturing elution using SDS sample buffer (all ALFA Selector Resins).

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

(Protocol continued on next page)

II. Analytical scale purifications using magnetic ALFA Selector Resins (continued)

Elution procedures:

a. **Peptide elution batch mode** (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 40 mg/ml in deionized water.
ii. Dilute the ALFA peptide stock solution 1:100 in physiological buffer to obtain an Elution buffer containing 200 µM ALFA elution peptide.
iii. Add 5 resin volumes (RV) Elution buffer to the resin.
iv. Incubate with subtle shaking:
ALFA Selector^{PE}: 15-20 min at room temperature.
ALFA Selector^{CE}: 3-5 min at room temperature.
or 15-20 min at 4°C.
v. Sediment the beads by placing on a magnetic rack and collect the eluate (= supernatant).
vi. **Optional:** For highest yields repeat steps iii-v. Combine the eluates.

b. **Elution under acidic conditions** (all ALFA Selector variants).

Please check the general remarks related to acidic elution approaches on page 8.

i. Prepare Acidic Elution Buffer (e.g. 0.1 M Glycine/HCl pH 2.2, 150mM NaCl).
ii. Add 2.5 resin volumes (RV) of Acidic Elution Buffer to the resin.
iii. Incubate for 2 min at RT with subtle shaking.
iv. Sediment the beads by placing on a magnetic rack and collect the eluate (= supernatant).
v. Repeat steps ii-iv once.
vi. Combine eluates.
vii. Neutralize eluates by adding e.g. 1/10 volume 1 M Tris-HCl pH 8.5.

c. **Denaturing elution using SDS sample buffer** (all ALFA Selector variants).

Denaturing elution will in general not be compatible with protein functionality. It is therefore explicitly recommended for analytical applications only.

i. Add 2.5 resin volumes (RV) of 2x SDS sample buffer pre-warmed to ~60°C to the resin.
ii. Incubate for 5 min at 80-90°C with subtle shaking.
iii. Sediment the beads by placing on a magnetic rack and collect the eluate (= supernatant).
iv. Repeat steps i-iii once.
v. Combine eluates.

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

14. Analyze collected samples by SDS-PAGE.

III. Preparative purifications using peptide elution in flow or stopped flow mode

Notes:

Peptide elution 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. Peptide elution in flow mode generally requires a fast release of target proteins, which can most efficiently be assured when eluting **ALFA Selector^{CE} at room temperature**. Flow elution from ALFA Selector^{CE} at 4°C is possible but will generally require flow rates <0.05 resin volumes (RV)/min[2].

Procedure:

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^{CE}:
   a. Resuspend ALFA Selector^{CE}.
   b. Transfer the slurry to a clean flow column.
   c. Wash resin with at least 5 resin volumes (RV) 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 flow column used in step 3.

b. **Binding in flow mode:**

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

5. Wash resin with 10-20 RV of Washing buffer.
6. Elution:
   a. Prepare a 100x (20 mM) stock solution of ALFA elution peptide by solubilizing the ALFA elution peptide at 40 mg/ml in deionized water.
   b. Dilute the ALFA peptide stock solution 1:40 in physiological buffer to obtain an Elution buffer containing 500 µM ALFA elution peptide.
   c. Elute column in flow mode at a flow rate of 0.1 to 0.2 RV/min (room temperature) or <0.05 RV/min at 4°C.

   Note: When using gravity flow columns, the elution speed can be adjusted by adding buffer aliquots at defined time intervals: E.g. to achieve an effective flow rate of 0.1 RV/min, add 0.2 RV of Elution buffer every 2 minutes and collect the eluate correspondingly. To lower the net flow rate, increase the intervals between each addition of Elution buffer.
7. Analyze input material, non-bound material and eluate fractions by SDS-PAGE.

Notes:

- A slightly enhanced elution efficiency can generally be observed when increasing the peptide concentration to 1 mM[2]. Even higher peptide concentrations will generally not result in sharper elution profiles.
- The highest target protein concentrations can be achieved at low elution speed[2].
- Target proteins displaying a single ALFA-tag are typically eluted within 1-2 resin volumes[2].

IV. Preparative purifications using acidic elution in flow or stopped flow mode

Please check the general remarks related to acidic elution approaches on page 8.

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:
   a. Resuspend ALFA Selector.
   b. Transfer the slurry to a clean flow column.
   c. Wash resin with at least 5 resin volumes (RV) 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 flow column used in step 3.

b. Binding in flow mode:

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

5. Wash resin with 10-20 RV of Washing buffer.
6. Elution:
   a. Elute the column using an Acidic Elution Buffer at a flow rate of 0.2-0.5 RV/min.
   b. Immediately neutralize all eluate fractions e.g. by adding 1/10 volume of 1M Tris pH 8.5.

Note: When using gravity flow columns, the elution speed can be adjusted by adding buffer aliquots at defined time intervals: E.g. to achieve a net flow rate of 0.2 RV/min, add 0.2 RV of Elution buffer every minute and collect the eluate correspondingly. To lower the net flow rate, increase the intervals between each addition of Acidic Elution Buffer.

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

V. Buffer compatibility [1,2]

Please note that the buffer compatibility of ALFA Selector Resins differs for binding and washing steps, respectively. In general, ALFA Selector Resins are less sensitive when charged with an ALFA-tagged target protein. Therefore, it is possible to perform highly stringent washing steps under conditions incompatible with an initial binding.

Not all possible combinations of reagents have been explicitly tested. It is likely that certain combinations of reagents show more than additive effects. For example, such non-linear effects can be expected when combining denaturing agents (including denaturing detergents like SDS) with high concentrations of reducing agents.

A. Compatibility during binding

Class	Reagent	ALFA SelectorST	ALFA Selector^{PE}	ALFA Selector^{CE}
Salt	NaCl	>3 M	>3 M	>3 M
	$MgSO_4$	>1 M	>1 M	0.8 M
Denaturing agents	Urea	1.5 M	1.5 M	1.5 M
	Guanidin-HCl	0.3 M	0.3 M	0.3 M
Detergents	Triton-X 100	0.5 %	0.5 %	0.5 %
	DDM	0.5 %	0.5 %	0.5 %
	CHAPS	0.5 %	0.5 %	0.5 %
	Desoxycholate	0.1 %	0.1 %	0.1 %
	SDS	not compatible	not compatible	not compatible
Reducing agents	DTT	>100 mM	>100 mM	>100 mM
pH value[(a)]		6.5 – 8.0	6.5 – 8.0	6.5 – 8.0
Other substances			not tested	

[(a)] We generally recommended binding at physiological pH. Binding beyond the given pH boundaries has to be tested on a case-to-case basis.

B. Compatibility during washing

Class	Reagent	ALFA SelectorST	ALFA Selector^{PE}	ALFA Selector^{CE}
Salt	NaCl	>3 M	>3 M	>3 M
	$MgSO_4$	>1 M	>1 M	0.8 M
Denaturing agents	Urea	6 M	3 M	1.5 M
	Guanidin-HCl	>2 M	2 M	0.3 M
Detergents	Triton-X 100	>1 %	>1 %	0.8 %
	DDM	>1 %	>1 %	0.8 %
	CHAPS	>1 %	>1 %	0.8 %
	Desoxycholate	0.5 %	0.5 %	0.1 %
	SDS	0.1 %[(b)]	not compatible	not compatible
Reducing agents	DTT	>100 mM	>100 mM	>100 mM
pH value		5 – 10	5 – 10	6 – 10
Other substances			not tested	

[(b)] Time- and temperature-dependent partial leakage. Not recommended for prolonged washing steps.

VI. Specific notes on acidic elution procedures

- Acidic elution can be performed with all ALFA Selector variants at room temperature or at 4°C. The ALFA Selector variants display slightly different sensitivity towards low pH (see table below).

Selector Variant	Stable binding	Efficient elution
ALFA SelectorST	pH 5 to pH 10	pH 2.2
ALFA Selector^{PE}	pH 5 to pH 10	pH 3 or below
ALFA Selector^{CE}	pH 6 to pH 10	pH 4 or below

- An efficient elution from all ALFA Selector variants can generally be achieved using an Acidic Elution Buffer containing 0.1 M Glycine/HCl pH 2.2, 150 mM NaCl.
- Not all target proteins will tolerate low pH values. It is therefore important to adjust the elution conditions to the target protein of choice.
- It may be required to optimize the flow rate/incubation time, temperature and pH value in order to maintain the proper folding/activity of the target protein and/or the integrity of purified protein complexes.

VII. References

(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.

(2) Kilisch M, Götzke H, Gere-Becker M, Crauel A, Opazo F, Frey S. **Discovery and Characterization of an ALFA-Tag-Specific Affinity Resin Optimized for Protein Purification at Low Temperatures in Physiological Buffer.** Biomolecules. 2021 Feb 12;11(2):269.

All ALFA Selector Resins are only for research applications, not for diagnostic or therapeutic use!

For further information concerning this protocol please contact us at
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