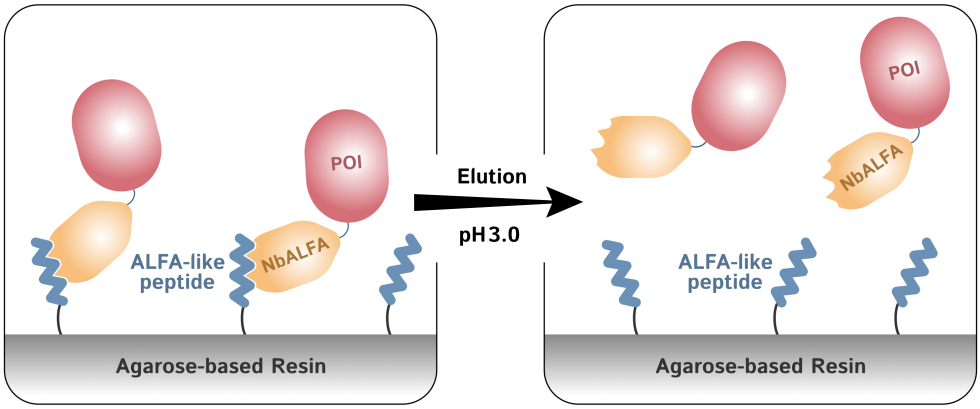
ALFA Peptide Resins for Purification of NbALFA Fusion Proteins

A. Introduction

The ALFA® system is based on the ALFA-tag, a highly versatile epitope tag with the core sequence SRLEEELRRRLTE, and its high-affinity binding partner, NbALFA (sdAb anti-ALFA), a single-domain antibody (sdAb) with a dissociation constant of approximately 26 pM .

ALFA Peptide Resins are affinity resins specifically designed for the selective purification of proteins fused to NbALFA. They are available in two alternative formats, based on 4 % cross-linked agarose or 4 % magnetic agarose. Both resins feature an immobilized ALFA-like peptide that enables the efficient and highly specific capture of NbALFA and NbALFA-tagged protein of interest (POI).

Elution is performed under mild acidic conditions (pH 3.0), which typically preserves the native conformation and functionality of sensitive proteins by avoiding harsh denaturing or reducing agents. This makes ALFA Peptide Resins particularly suitable for applications requiring functional, structurally intact proteins.



The high specificity of ALFA Peptide Resins arises from the strong and selective interaction between the immobilized ALFA-like peptide and NbALFA fused to the target protein. This ensures minimal non-specific binding and robust, reproducible purification results. Both available resin variants are compatible with various buffer compositions during binding and washing and tolerate high concentrations of common detergents and reducing agents (see Compatibility Chart on page 8), offering flexibility for customized purification workflows.

Due to the covalent attachment of the ALFA-like peptide, the resins can withstand multiple regeneration cycles and harsh cleaning-in-place (CIP) procedures without significant loss of performance.

Formats and Applications

ALFA Peptide Resins are available in two alternative formats:

Product Name	Cat. No.	Bead Format	Handling Options
ALFA Peptide Resin 10 mL, 50 % slurry	N1530	4 % cross-linked agarose	Suitable for standard chromatography applications including batch purifications and column formats operated by gravity flow or FPLC
ALFA Peptide Resin, magnetic 10 mL, 50 % slurry	N1535	4 % magnetic agarose	Optimized for magnetic bead separation protocols

Capacity

The effective binding capacity of ALFA Peptide Resins depends on the hydrodynamic properties of the proteins being purified – such as their size and shape. Due to size exclusion effects, the capacity may be reduced for large proteins or protein complexes. The table below provides representative capacities for two selected example proteins.

Protein (Cat. No.)	Molecular Weight	Binding Capacity
NbALFA (N1500)	14 kDa	~500 µM (~7 mg/mL)
NbALFA-Halo fusion (N1541)	48 kDa	~400 µM (~19 mg/mL)

Distinguishing ALFA Peptide Resins from ALFA Selector Resins

It is essential to clearly distinguish between ALFA Peptide Resins and ALFA Selector Resins, as they are designed for fundamentally different applications. The table below summarizes the key features that differentiate the two product types.

Product	Cat. No.	Resin	Purpose
ALFA Peptide Resins	N1530, N1535	Resins feature immobilized ALFA-like peptide	Purification of NbALFA fusion proteins
ALFA Selector Resins	N1510, N1511, N1512, N1515, N1516, N1517	Resins feature immobilized NbALFA variants	Purification of ALFA-tagged proteins

B. Protocols

I. Required Materials (not included)		
Buffers	Lysis Buffer[a]	PBS or TBS (other formulations possible)[a]
	Washing Buffer[a]	PBS or TBS (other formulations possible)[a]
	Acidic Elution Buffer[b]	0.1 M sodium citrate pH 3.0, 150 mM NaCl
	Neutralization Buffer	1.5 M Tris/HCl pH 8.8
	2x SDS Sample Buffer	125 mM Tris/HCl pH 6.8, 4% SDS, 100 mM DTT, 1 M sucrose, 0.1 mg/mL bromophenol blue
Columns and Tubes		
Optional: Magnetic rack		

[a] Buffers can be adjusted across a wide range of conditions. See the buffer compatibility chart on page 8.
[b] Refer to the specific notes on acidic elution procedures on page 8.

II. Analytical purifications using non-magnetic ALFA Peptide Resin

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

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

1. Prepare cell lysates (0.2 to 1.5 mL volume) from cells expressing an NbALFA fusion protein according to established protocols. For mammalian cells, we recommend using 10^6-10^8 cells per experiment. NbALFA fusion proteins may alternatively be purified from cell culture supernatants.
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 Peptide Resin:
   a. Resuspend ALFA Peptide 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.
4. Add cleared lysate from step 2 to the equilibrated ALFA Peptide 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 MiniSpin Column. Place column in 2 mL reaction tube.
   b. Resuspend beads in 200 µL Lysis Buffer and transfer suspension to MiniSpin Column.
   c. Wash out remaining beads sticking to the tube with 200 µL Lysis Buffer.
      Transfer the suspension to the MiniSpin 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.
10. Wash with 400 µL TBS, centrifuge for 1 min at 1000 x g.
11. Attach bottom plug and place MiniSpin Column in a clean 1.5 mL reaction tube.

(Protocol continued on next page)

12. Elution:

a. Elution under acidic conditions

NbALFA fusion proteins can generally efficiently be eluted under acidic conditions. We recommend using 0.1 M sodium citrate pH 3.0, 150 mM NaCl as the Acidic Elution Buffer.

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

i. Add 2.5 resin volumes (RV) of Acidic Elution Buffer to the resin.
ii. Incubate for 2 min at RT with subtle shaking.
iii. Remove the bottom plug and collect the eluate by centrifugation 1 min at 1000 x g.
iv. Attach bottom plug and repeat steps i-iii.
v. Combine eluates.
vi. Neutralize eluates by adding 1/10 volume 1.5 M Tris-HCl pH 8.8.

b. Denaturing elution using SDS Sample Buffer

NbALFA fusion proteins can also be eluted using SDS Sample Buffer at elevated temperatures. 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. Close the MiniSpin 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 i-iv.
vi. Combine eluates.

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

14. Analyze collected samples by SDS-PAGE.

III. Analytical purifications using magnetic ALFA Peptide Resin

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

1. Prepare cell lysates (0.2 to 1.5 mL volume) from cells expressing an NbALFA fusion protein according to established protocols. For mammalian cells, we recommend using 10^6-10^8 cells per experiment. NbALFA fusion proteins may alternatively be purified from cell culture supernatants.
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 Peptide Resin:
   a. Resuspend ALFA Peptide 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. Place the tube in a magnetic rack. Wait until beads are settled (>2 min).
   e. Remove supernatant.
   f. Repeat steps 3c-3e.
4. Add cleared lysate from step 2 to the equilibrated magnetic ALFA Peptide 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 with 1 mL TBS and collect beads on magnetic rack.
   i. Remove the supernatant completely.

(Protocol continued on next page)

8. Elution:

a. Elution under acidic conditions

NbALFA fusion proteins can generally efficiently be eluted under acidic conditions. We recommend using 0.1 M sodium citrate pH 3.0, 150 mM NaCl as the Acidic Elution Buffer.

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

i. Add 2.5 resin volumes (RV) of Acidic Elution Buffer to the resin.
ii. Incubate for 2 min at RT with subtle shaking.
iii. Sediment the beads by placing on a magnetic rack and collect the liquid phase (eluate).
iv. Repeat steps i-iii.
v. Combine eluates.
vi. Neutralize eluates by adding 1/10 volume 1.5 M Tris-HCl pH 8.8.

b. Denaturing elution using SDS Sample Buffer

NbALFA fusion proteins can also be eluted using SDS Sample Buffer at elevated temperatures. 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.
iv. Collect the liquid phase (eluate).
v. Repeat steps i-iv.
vi. Combine eluates.

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

10. Analyze collected samples by SDS-PAGE.

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 from cells expressing an NbALFA fusion protein according to established protocols. NbALFA fusion proteins may alternatively be purified from cell culture supernatants.
2. Clear lysate by centrifugation for 10 min at > 14000 x g and 4 °C.
3. Equilibrate ALFA Peptide Resin:
   a. Resuspend ALFA Peptide Resin.
   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:

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

b. Binding in flow mode:

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

5. Wash resin with 10-20 RV of Washing Buffer.
6. Elution:

Elute the column using Acidic Elution Buffer at a flow rate of 0.2-0.5 RV/min.

Immediately neutralize all eluate fractions by adding 1/10 volume of 1.5 M Tris pH 8.8.

Note: When using gravity flow columns, the elution speed can be adjusted by adding buffer aliquots at defined time intervals. As an example: 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.

C. Appendix

I. Buffer compatibility [1,2]

Please note that the buffer compatibility of ALFA Peptide Resins differs for binding and washing steps, respectively. In general, NbALFA is more robust when bound to ALFA Peptide Resins. Therefore, it is possible to perform highly stringent washing steps under conditions incompatible with an initial binding.

Not all reagent combinations are compatible and some may exhibit more than additive – i.e., synergistic or non-linear – effects. For example, such effects might be expected when combining denaturing agents (including denaturing detergents like SDS) with high concentrations of reducing agents.

Class	Reagent	During Binding	During Washing
Salt	NaCl	>3 M	>3 M
	$MgSO_4$	0.8 M	0.8 M
Denaturing agents	Urea	1.5 M	1.5 M
	Guanidin-HCl	0.3 M	0.3 M
Detergents	Triton-X 100	0.5 %	0.8 %
	DDM	0.5 %	0.8 %
	CHAPS	0.5 %	0.8 %
	Desoxycholate	0.1 %	0.1 %
	SDS	not compatible	not compatible
Reducing agents	DTT	>100 mM	>100 mM
pH value[(a)]		6.5 – 8.0	5 – 10
Other substances		not tested	

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

II. Specific notes on acidic elution procedures

- Acidic elution is compatible with all ALFA Peptide Resin variants and can be performed at room temperature or 4 °C.
- An efficient elution is generally achieved using an Acidic Elution Buffer consisting of 0.1 M sodium citrate, pH 3.0, and 150 mM NaCl.
- Not all proteins tolerate low pH. It is essential to optimize elution conditions based on the properties of the target protein. For highly sensitive proteins, consider eluting at **pH 3.5** using 0.1 M sodium citrate, pH 3.5, 150 mM NaCl. Note that elution efficiency or kinetics may be reduced at this pH.
- For particularly robust proteins or in cases of poor elution efficiency, using 0.1 M glycine/HCl, **pH 2.2**, 150 mM NaCl may improve recovery.
- Optimization of elution parameters – including flow rate, incubation time, temperature, and pH – may be necessary to preserve protein folding, activity, or the integrity of multi-protein complexes.

III. Regenerating ALFA Peptide Resins, Cleaning in Place (CIP)

ALFA Peptide Resins are chemically robust and can be regenerated under harsh conditions for at least 10 cycles without significant loss of performance. Recommended regenerations protocols include:

- 100 mM NaOH, 15 min at room temperature
- 100 mM HCl, 15 min at room temperature
- 6 M guanidinium-HCl, 15 min at room temperature

For long-term storage, transfer the resin in PBS containing 20 % ethanol.

IV. 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 Peptide Resins are only for research applications, not for diagnostic or therapeutic use!

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