5.2 Protein purification

5.2.1 Purification of a His_6-tagged Green Fluorescent Protein (GFP)

Principle

You can add either a N- or C-terminal His_6-tag to the protein that you want to express if you use the RTS pIVEX His_6-tag 2nd generation vector set (pIVEX2.3d; pIVEX2.4d, see Chapter 2.4.2.1) or the RTS *E. coli* Linear Template Generation Set, His_6-tag (see Chapter 2.3.3.1).

These His_6-tagged proteins can be purified in one step by immobilized metal affinity chromatography (IMAC) (Ford, C. F. et al., 1991) on a nickel-nitrilotriacetic acid (Ni-NTA) column. In a single step, this affinity matrix can purify a protein (starting concentration less than 1% of the total protein) to more than 95% homogeneity.

Nitrilotriacetic acid (NTA) is a tetradentate chelating adsorbent developed by Roche Diagnostics GmbH. NTA occupies four of six ligand binding sites of the nickel ion, leaving two sites free for interaction with the His_6-tag. NTA binds metal ions tightly, allowing use of stringent washes.

Histidine residues on the tag, connected via a short linker to the C- or N-terminus of the protein, bind to the Ni-ions. The protein can be eluted by competitive displacement with imidazole.

***Note*:** Since Ni-NTA is not as selective as other affinity chromatography matrices, it may also bind proteins with exposed patches of histidine, cysteine or tryptophan residues. Therefore elution conditions must be optimized for each protein. An easy way to optimize conditions is to use an imidazole gradient for elution, rather than a single imidazole concentration.

Protocol

The following purification protocol is optimized for purification of His_6-tagged GFP. If it is used to purify other proteins, the protocol may have to be modified. For more detailed information see the manufacturer's handbook provided with the purification matrix.

Material required

Reagent	Vendor
Ni-NTA agarose	QIAgen
1 ml column with luer lock on both ends	MoBiTec
10 ml luer lock syringe	Merck Eurolab

Buffer	Composition
Equilibration buffer	20 mM Tris/HCl, 200 mM NaCl; pH 7.5
Washing buffer	20 mM Tris/HCl, 200 mM NaCl, 5 mM imidazole; pH 7.5
Elution buffer 1*	20 mM Tris/HCl, 200 mM NaCl, 20 mM imidazole; pH 7.5
Elution buffer 2*	20 mM Tris/HCl, 200 mM NaCl, 200 mM imidazole; pH 7.5
Elution buffer 3	20 mM Tris/HCl, 200 mM NaCl, 500 mM imidazole; pH 7.5

**The imidazole concentrations of elution buffers 1 and 2 must be optimized for each protein.*

Notes on use of Ni-NTA column:

- We recommend using new Ni-NTA resin for each purification.
- Use only gravity flow for all purification steps.
- Perform all steps at 4°C.
- Before mounting the luer lock syringe on top of the column, make sure that the column is filled to the top with buffer and that all air bubbles remain above the resin bed. Remove these air bubbles with a syringe or thin pipette tip.
- When changing the buffer:
  - Let the current buffer flow through the column until the buffer reservoir is nearly empty.
  - Add only a small volume of the new buffer.
  - Let that volume flow through the column until the reservoir is nearly empty again.
  - Finally, begin washing the column extensively with the new buffer.
- Make sure that the column does not run dry at any point in the procedure.

Procedure for purification of a His_6-tagged protein

Step	Action
1	▶ Fill the column with Ni-NTA resin to create a bed volume of 0.6 ml. ▶ Close the column and mount the luer lock syringe (without plunger) as a buffer reservoir. ▶ Equilibrate the column with 10 to 15 bed volumes (6 – 9 ml) of equilibration buffer.
2	▶ Apply the sample to the column by gravity flow. Keep a small portion of the sample for assays (in Step 4). ***Note*:** Often, you can apply the contents of the RTS reaction chamber directly to the column. However, if you see precipitate in the sample that might clog the column, centrifuge the sample at 10 000 x *g* for 1 min to remove the precipitate before applying the sample to the column.
3	▶ Immediately after the sample has entered the resin, wash the column with 10 bed volumes (6 ml) washing buffer.
4	▶ Beginning with the first washes, collect 1 ml fractions of effluent from the column throughout the entire purification. ▶ Monitor the progress of the purification by analyzing each fraction by SDS-PAGE, Western blotting and/or activity assay. Use the unpurified sample as a reference in these assays.
5	▶ Elute nonspecifically bound proteins with 10 bed volumes (6 ml) elution buffer 1. ***Note*:** The imidazole concentration in elution buffer 1 must be optimized for each protein.
6	▶ Elute specifically bound protein with 10 bed volumes (6 ml) elution buffer 2. ***Note*:** The imidazole concentration in elution buffer 2 must be optimized for each protein. For GFP with the His_6-tag on either end, 200 mM imidazole showed the best results.
7	▶ After all specifically bound protein has been eluted from the column, wash the column with 10 bed volumes (6 ml) elution buffer 3. This will elute all bound proteins from the column.

Typical result

Figure 57 shows an SDS-PAGE assay of the purification of GFP with a C-terminal His_6-tag. The recovery of purified protein was about 96%. Of that total, 94% emerged in the first two fractions eluted with elution buffer 2 (Figures 57 and 58).

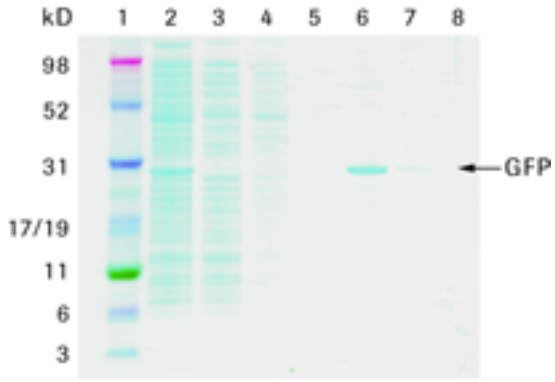

Figure 57: Purification of GFP with a C-terminal His_6-tag: Assay of eluted fractions by 10% Bis/Tris (MOPS) SDS-PAGE.
1: Molecular weight standard; 2: Crude extract; 3: Flow-through; 4: Pooled washing fractions; 5: Elution 1 with 20 mM imidazole, pooled; 6-8: Elution 2 with 200 mM imidazole, fractions 1, 2, and 3 respectively.

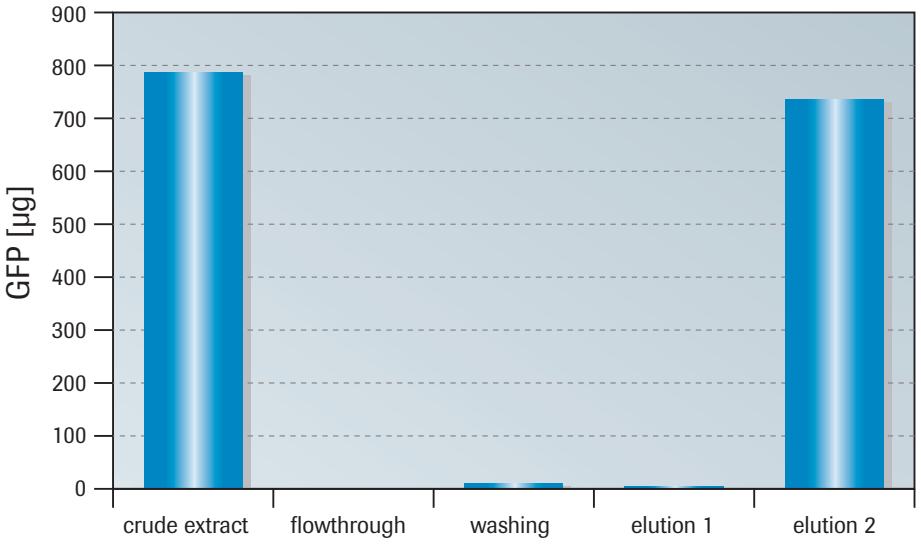

Figure 58: Recovery of His_6-tagged GFP during the purification procedure.

5.2.2 Purification of an MBP fusion protein

Principle

To increase the solubility of a protein, express it with an N-terminal MBP fusion. This fusion can be added with either the RTS *E. coli* Linear Template Generation Set, MBP Fusion (see Chapter 2.3.3.4) or the pIVEX-MBP cloning vector (see Chapter 2.4.2.3).

The expressed fusion protein can then be purified in one step by affinity chromatography on amylose matrices (Maina, C.V. et al., 1988). The maltose binding protein, connected via a short linker to the N-terminus of the desired protein, binds to the amylose resin. The protein can be eluted by competitive displacement with maltose. The protein may also be cleaved with Factor Xa protease (see Chapter 5.3), either while it is still bound to the column or after it is eluted.

The fusion protein carries an additional N-terminal His_6-tag that would allow one-step purification by Ni-NTA affinity chromatography (see Chapter 5.2.1).

Protocol

The following purification protocol is optimized for the purification of MBP-endoglycosidase. If it is used to purify other proteins, the protocol may have to be modified. For more detailed information see the manufacturer's handbook provided with the purification matrix.

Note: The resin can be reused three to five times if it is regenerated according to the manufacturer's manual.

Material required

Reagent	Vendor
Amylose resin	New England Biolabs
1 ml column with luer lock on both ends	MoBiTec

Buffer	Composition
Equilibration buffer	10 mM Tris-HCl; pH 7.2
Wash buffer	10 mM Tris-HCl, 1 M NaCl; pH 7.2
Elution buffer	10 mM Tris-HCl, 10 mM maltose; pH 7.2

Procedure

Step	Action
1	▶ Perform the following steps at 4°C, using only gravity flow.
2	▶ Pour the appropriate amount of amylose resin into the column. ***Note:*** In general, 1 ml resin is enough for one RTS 500 *E. coli* HY reaction. The binding capacity of amylose resin is approx. 3 mg/ml. ▶ Equilibrate the column with 8 column volumes of equilibration buffer.
3	▶ Centrifuge the sample at 10 000 x g for 1 min to remove any precipitated protein that might clog the column. ▶ Apply the supernatant to the column by gravity flow. Keep a small portion of the supernatant for assays (in Step 6). ▶ Once all the sample has entered the column, shut off the column flow.
4	▶ Incubate the column (containing the sample) for 15 min to enable optimal binding between the fusion protein and the amylose resin.
5	▶ Wash the column with 10 bed volumes of wash buffer.
6	▶ Beginning with the first washes, collect fractions of effluent from the column throughout the entire purification. ***Note:*** Fraction size should be 1/3 of column volume. ▶ Monitor the progress of the purification by analyzing each fraction by SDS-PAGE, Western blotting and/or activity assay. Use the unpurified sample as a reference in these assays.
7	▶ Elute bound protein with 2–4 bed volumes of elution buffer. ***Note:*** The fusion protein usually starts to elute within the first 5 fractions. It should easily be detected by UV absorption at 280 nm or the Bradford protein assay.

Typical result

Figure 59 shows an SDS-PAGE assay of the purification of MBP-endoglycosidase on amylose resin. Staining was done with SimplyBlue Safe Stain (Invitrogen). The additional bands in lanes 9 and 10 are degradation products of the MBP fusion protein.

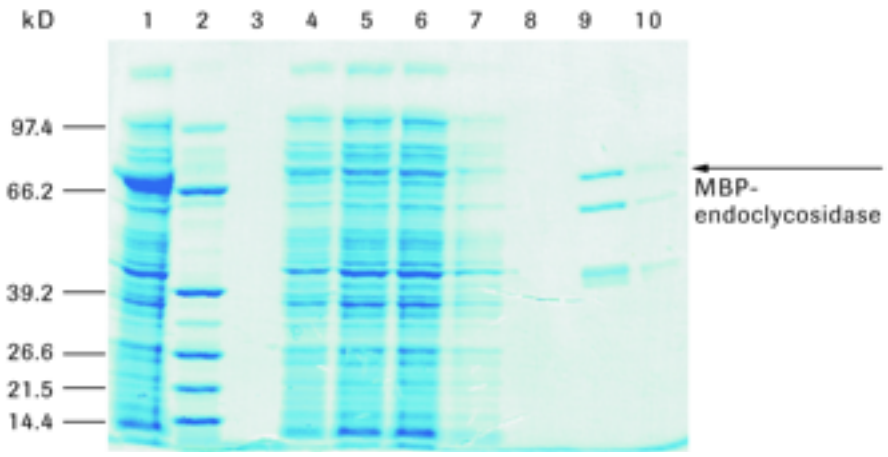

Figure 59: Purification of MBP-endoglycosidase:
Assay of eluted fractions by 10% Bis/ Tris (MOPS) SDS-PAGE.
1: Supernatant of reaction solution; 2: Molecular weight standard; 3–5: Flow-through; 6–7: Washing fractions; 8–10: Elution fractions; 10 µl of sample were loaded on each lane.

5.2.3 Purification of an HA-tagged protein

Principle

In some cases, you may need an alternative tag. You can use either the RTS *E. coli* Linear Template Generation Set, HA-tag (see Chapter 2.3.3.2), or the vectors pIVEX2.5d and pIVEX2.6d (see Chapter 2.4.2.2) to attach either a C-terminal HA-tag (pIVEX2.5d) or an N-terminal HA-tag (pIVEX2.6d) to the protein that you want to express.

These HA-tagged proteins can be purified in one step by affinity chromatography on an Anti-HA Affinity Matrix. The Anti-HA Affinity Matrix is also suitable for:

- Affinity purification of HA-tagged proteins from crude protein extracts.
- Immunoprecipitation of HA-tagged proteins from mammalian, yeast and bacterial cell extracts.

The purified protein can be analyzed on a Western blot with an Anti-HA antibody.

Protocol

The following purification protocol is optimized for the purification of an HA-tagged GFP mutant. If it is used to purify other proteins, the protocol may have to be modified. For further details see the pack insert of the Anti-HA Affinity Matrix (Cat. No. 1 815 016).

Material required

Reagent or equipment	Vendor	Cat. No.
Anti-HA Affinity Matrix	Roche Applied Science	1 815 016
HA peptide	Roche Applied Science	1 666 975
1 ml column with luer lock on both ends	MoBiTec	
23-gauge (23 G) needle for adjustment of flow rate ***Note:*** This needle size is suitable for a 0.5–1.0 ml column. Adjust needle size as needed for larger or smaller columns.		

Buffer*	Composition
Equilibration buffer	20 mM Tris-HCl, 0.1 M NaCl, 0.1 mM EDTA; pH 7.5
Wash buffer	20 mM Tris-HCl, 1 M NaCl; pH 7.5
Elution buffer	Dissolve HA peptide at 1 mg/ml in equilibration buffer. ***Note:*** Store at –20°C and thaw before use.
Column storage buffer	20 mM Tris, 0.1 M NaCl, 0,1 mM EDTA, 0.09% sodium-azide; pH 7.5
Regeneration buffer	0.1 M Glycine, pH 2.0

** 100 ml of each buffer should be enough to purify several expressed proteins. All buffers except the elution buffer may be stored at 2°–8°C for up to one month (store the elution buffer at –20°C). Bring buffers to room temperature before use.*

Procedure

A. Preparing the column

Step	Action
1	▶ Perform all the steps of the purification at 4°C unless noted otherwise. Use only gravity flow to elute the column.
2	▶ Attach lower cap to bottom of column, and place column on rack or stand above a collection tube.
3	▶ Gently invert Anti-HA Affinity Matrix several times to thoroughly resuspend beads.
4	▶ Pipet desired volume of slurry into column. ***Note:*** We suggest a final settled bead volume of 0.5–1.0 ml.
5	▶ Replace lower cap with 23 G needle.
6	▶ Drain column storage buffer into collection tube. ***Note:*** Do not allow column to dry out. Flow rate should be approximately 0.3–0.5 ml/minute. If flow rate is faster, use a smaller gauge needle to adjust the rate.
7	▶ Immediately add 10 bed volumes of equilibration buffer and allow buffer to drip through column.

B. Loading the column

Step	Action
1	▶ Determine amount of crude protein extract (RTS reaction solution) to be purified. ***Notes:*** ▶ Optimal sample volume is 1–4 ml for a 1 ml column. ▶ Amount of total protein in RTS reaction solution is typically 1–5 mg. However, the total protein concentration of the solution depends upon expression levels of tagged protein.
2	▶ Centrifuge the sample at 10 000 x g for 1 min to remove any precipitated protein that might clog the column. ***Note:*** Column may become fouled if excessive total protein is loaded or insoluble materials are not completely removed from the RTS reaction solution. ▶ Apply the supernatant to the equilibrated column by gravity flow.
3	▶ Collect flow-through in a clean collection tube. Save this crude protein extract fraction at 4°C.

C. Eluting proteins

Step	Action
1	▶ Wash column with a minimum of 20 bed volumes of washing buffer at room temperature to remove nonspecifically bound protein. ***Note:*** Read the OD_{280} of the effluent at the end of this step to verify that the final wash fractions contain no protein and are close to baseline levels (= wash buffer alone). ▶ Save wash fractions if desired.
2	▶ Immediately replace needle with lower column cap. ▶ Add 1 bed volume of elution buffer to the column and incubate for 15 minutes at 37°C. ***Note:*** Elutions may be performed at a lower temperature; however this will lower the yield of purified protein.
3	▶ Replace the lower cap with the needle and collect the elution fraction in a clean collection tube (microcentrifuge tube or equivalent). ▶ Keep samples cold (4°C) until they are analyzed.
4	▶ Repeat Steps 2 and 3 twice.
5	▶ Read OD_{280} for each fraction and pool as desired. ***Note:*** The yield of purified protein depends upon expression levels. Typical results from a 1 ml column range from 2 to 8 nmol of purified protein.

D. Column reuse, regeneration and storage

Step	Action
1	▶ Strip column by running 20 bed volumes of regeneration buffer through the column. ***Note***: Depending on the protein used, complete regeneration of the matrix may require up to 160 bed-volumes of regeneration buffer.
2	▶ Immediately re-equilibrate column with 20 bed volumes of equilibration buffer.
3	▶ Store column tightly capped at 4°C in 2 bed volumes of column storage buffer. ***Note:*** The affinity matrix may be used at least ten times. Suitability of the column for more than ten uses should be determined by the user and will depend upon the tagged protein or cell extract used.

Typical result

Figure 60 shows an SDS-PAGE assay of the purification of an HA-tagged protein on an anti-HA matrix. The gel was stained with Coomassie Blue.

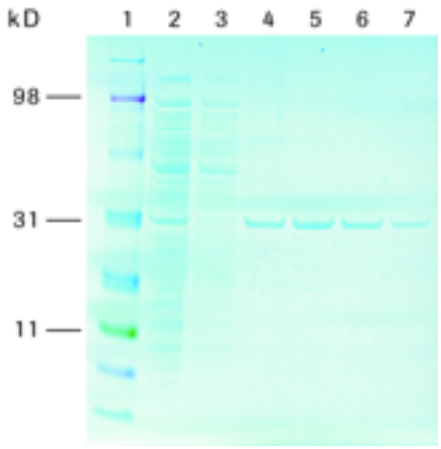

Figure 60: 10% Bis/Tris (MOPS) SDS-PAGE illustrating the purification of an HA-tagged GFP mutant.
1: Molecular weight standard, 10 µl; 2: pIVEX 2.6 GFP mutant expression, supernatant; 3: Wash fraction; 4: Elution step 1; 5: Elution step 2; 6: Elution step 3; 7: Elution step 4; Samples loaded on the gel were 0.5 µl pIVEX 2.6 GFP mutant expression supernatant (2), 5 µl wash fraction (3) and elutions (4, 5, 6, 7).

5.2.4 Troubleshooting the purification procedures

Observation	Possible cause	Recommendation
His_6-tag		
Fusion protein does not bind to the column.	His_6-tag not present	▶ Check sequence and reading frame. ▶ Check for possible internal translation starts (N-terminal tags) or premature termination sites (C-terminal tags).
	Binding conditions incorrect	▶ Check that the correct buffers and pH have been used. ▶ Decrease the concentration of imidazole in the binding buffer. ▶ Ensure that there are no chelating or reducing agents present. ▶ If reusing a column, make sure it has been regenerated correctly.
	Tag may be inaccessible.	▶ Put tag on another part of the protein. ▶ Purify protein under denaturing conditions (*e.g.*,sodium phosphate buffer, pH 8.0 with 8 M urea or 6 M guanidinium hydrochloride; optionally, add 10-500 mM imidazole to increase binding specificity).
	Tag may be degraded.	▶ Include protease inhibitors and perform purification at 4°C.
	Column capacity is exceeded	▶ Apply less fusion protein to column.
Protein elutes in the wash buffer.	Wash stringency too high	▶ Lower the concentration of imidazole or increase pH.
	His_6-tag partially hidden	▶ Reduce wash stringency. ▶ Purify under denaturing conditions.
Fusion protein elutes poorly.	Column may not have been correctly charged with nickel ions.	▶ Repeat column preparation steps.
	Elution conditions too mild	▶ Increase concentration of imidazole in the elution buffer (>400 mM). ▶ Use gradient with increasing concentrations of imidazole. ▶ Carefully lower pH to create more stringent elution conditions. ***Note:*** Do not use a pH below pH 3.5 because low pHs will strip metal ions off the column.
	Fusion protein may be precipitating.	▶ Purify protein at room temperature or at 4°C: Fill the column with elution buffer, incubate overnight and elute the protein with elution buffer the following day. ▶ Add solubilizing reagents, *e.g.,* 2 M NaCl, 50 mM CHAPS, 50% glycerol, 8 M urea, 6 M guanidine hydrochloride, 0.1-2% Tween 20, 0.1–2% Triton X-100. ***Note:*** Triton X-100 has a high absorbance at 280 nm, and cannot be removed by buffer exchange procedures. ▶ Add reducing agents such as 2-mercaptoethanol to help solubilization. ▶ Perform binding and elution in batch format to avoid high local concentrations of protein.
Protein elutes with contaminants.	Binding and wash conditions not stringent enough	▶ Use more stringent binding or washing conditions.
	Contaminants associated with tagged protein.	▶ Increase salt and/or detergent concentration or add glycerol to wash buffer to disrupt nonspecific interactions.
	Column too large	▶ Reduce amount of Ni-NTA matrix used.
	Contaminants are truncated forms of tagged protein.	▶ Check sequence for possible internal translation starts (C-terminal tags) or premature termination sites (N-terminal tags).
Discoloration of resin	Nickel ions are removed or reduced.	▶ Ensure that there are no chelating agents (which turn resin white) or reducing agents (which turn resin brown) present in the buffers.

HA-tag		
Little or no HA-tagged protein is eluted.	Tagged protein is degraded.	▶ Include protease inhibitors and perform purification at 4°C.
	Tagged protein not fully eluted	▶ If working at less than 37°C, increase temperature. ▶ Alternatively, increase time and/or volume of elution buffer. ▶ Try batch mixing of peptide solution with matrix.
	No or low expression of tagged protein	▶ Load larger volume of extract. ▶ Run column several times.
Large quantities of tagged protein present in the flow-through sample	Column is overloaded	▶ Decrease amount of loaded protein extract.
	Column not regenerated after last use	▶ Regenerate column before repurifying protein.
Tagged protein appears degraded (smear or multiple lower molecular weight bands seen on Western blot).	Protease activity during procedure	▶ Increase protease inhibitors in protein sample. ▶ Perform all steps at a lower temperature.
MBP Fusion		
Little or no MBP-tagged protein is eluted.	Tagged protein not fully eluted	▶ Try higher concentration of maltose (up to 500 mM). ▶ Try batch mixing of solution with matrix.