For life science research only.
Not for use in diagnostic procedures.

Anti-Protein C Affinity Matrix
from mouse $IgG_1\kappa$ (clone HPC4)

Version: 10
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Mouse monoclonal antibody (Clone HPC4), immobilized.

Cat. No. 11 815 024 001 1 ml
settled resin volume

Store the product at +2 to +8°C.

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1. General Information

1.1. Contents

Vial / bottle	Label	Function / description	Content
1	Immobilized Anti-Protein C	▪ Antibody is covalently coupled to the matrix (agarose beads) and is supplied as a 2 ml slurry containing 1 ml of beads in 1 ml of buffer (20 mM Tris, 0.1 M NaCl, 1 mM $CaCl_2$, and 0.09% sodium azide (w/v) as preservative). ▪ 4 mg of antibody is reacted per 1 ml of beads in the coupling reaction. ▪ One plastic column with top and bottom caps is included.	1 vial, 1 ml matrix

1.2. Storage and Stability

Storage Conditions (Product)

When stored at +2 to +8°C, the product is stable through the expiry date printed on the label.

Vial / bottle	Label	Storage
1	Immobilized Anti-Protein C	Store at +2 to +8°C. ⚠ ***Do not freeze.***

1.3. Additional Equipment and Reagent required

For affinity purification

See section, **Working Solution** *for additional reagents and information on preparing solutions.*

- Rack or stand suitable for gravity collection of samples
- 23G needle for adjustment of flow rate.
  This needle size is appropriate for 0.5 to 1.0 ml column bed volumes, but may be adjusted for larger or smaller columns.
- Equilibration buffer
- Wash buffer
- EDTA elution buffer
- Peptide elution buffer
- Column storage buffer
- Regeneration buffer

For immunoprecipitation

See section, **Working Solution** *for additional reagents and information on preparing solutions.*

- Microcentrifuge and 1.5 ml microcentrifuge tubes
- Pipette tips (wide-bore and small-bore types)
- End-over-end rocker
- Cell lysis buffer
- Wash buffer
- EDTA elution buffer

For western blotting

See section, **Working Solution** *for additional reagents and information on preparing solutions.*

- PVDF Western Blotting Membranes*
- Anti-Protein C
- Western Blocking Reagent*
- Lumi-Light Western Blotting Substrate*
- Lumi-Film Chemiluminescent Detection Film*
- Plastic wrap
- Transfer buffer
- Blocking buffer
- TBS-Ca^{2}+ solution
- TBST-Ca^{2}+ solution

1.4. Application

Anti-Protein C Affinity Matrix is suitable for:

- Affinity purification of Protein C-tagged proteins from crude protein extracts.
- Immunoprecipitation of Protein C-tagged proteins.

Following purification, the tagged protein of interest may be analyzed by:

- Western blotting using the anti-Protein C antibody.
- Silver staining or similar protein stain.

2. How to Use this Product

2.1. Before you Begin

Sample Materials

Affinity purification

Prepare protein extracts containing the Protein C-tagged protein of interest using any of a variety of standard methods. The following lysis buffers have performed well:

- Bacterial extracts: 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$, Complete Protease Inhibitor Cocktail Tablets, EDTA-free*
- Mammalian extracts: 50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 0.05% deoxycholate, 1 mM $CaCl_2$, Complete Protease Inhibitor Cocktail Tablets, EDTA-free*

Other cell lysis buffers may be more appropriate for individual applications. Avoid phosphate buffers to prevent precipitation.

General Considerations

To obtain optimal performance of the affinity column:

- Remove insoluble particulates from the protein extract prior to loading on the column. If insoluble particulates are present, remove particulates by centrifugation or by filtration.
- Add $CaCl_2$ stock solution to protein extract solution to obtain a final concentration of 1 mM Ca^{2}+.
- Use protease inhibitors, such as the Complete Protease Inhibitor Cocktail Tables, EDTA-free to reduce proteolytic activity in most applications.

  ⚠ ***Avoid EDTA which inhibits antibody binding.***
- Limit detergents to the lowest possible concentration levels necessary to obtain adequate cell lysis.
- Avoid overloading the column, which could result in decreased purity of the final product.

Working Solution

Affinity purification

Prepare approximately 100 ml of the following reagents before beginning the affinity purification.

Buffer	Composition/Preparation
Equilibration buffer	20 mM Tris*, pH 7.5, 0.1 M NaCl, 1 mM $CaCl_2$ ($CaCl_2$ stock solution: 1 M $CaCl_2$)
Wash buffer	20 mM Tris*, pH 7.5, 0.1 M NaCl, 1 mM $CaCl_2$
EDTA elution buffer	20 mM Tris*, pH 7.5, 0.1 M NaCl, 5 mM EDTA
Peptide elution buffer (optional)	20 mM Tris*, pH 7.5, 0.1 M NaCl, 1 mM $CaCl_2$, 0.5 mg/ml synthetic protein C peptide
Column storage buffer	20 mM Tris*, pH 7.5, 0.1 M NaCl, 1 mM $CaCl_2$, 0.09% sodium azide
Regeneration buffer (optional)	0.1 M glycine, pH 2.0

Immunoprecipitation

Prepare the following reagents before beginning the immunoprecipitation procedure.

Buffer	Composition/Preparation
Cell lysis buffer	50 mM Tris*, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1% Nonidet P-40*, 0.5% deoxycholate, cOmplete Protease Inhibitor Cocktail Tablets, EDTA-free*. *Other buffers may be substituted.*
Wash buffer	50 mM Tris*, pH 7.5, 250 mM NaCl, 1 mM $CaCl_2$, 0.1% Nonidet P-40*, 0.05% deoxycholate
EDTA elution buffer	20 mM Tris*, pH 7.5, 0.1 M NaCl, 5 mM EDTA

Western blotting

Prepare the following reagents before beginning the western blotting procedure.

Buffer	Composition/Preparation
Transfer buffer	10% methanol, 24 mM Tris base*, 194 mM glycine
Blocking buffer	Tris-buffered saline (10 mM Tris*, pH 7.5, 150 mM NaCl), containing 1 mM $CaCl_2$ and 1x Western Blocking Reagent*
TBS-Ca^{2}+ solution	Tris-buffered saline (10 mM Tris*, pH 7.5, 150 mM NaCl), containing 1 mM $CaCl_2$
TBST-Ca^{2}+ solution	Tris-buffered saline (10 mM Tris*, pH 7.5, 150 mM NaCl), containing 1 mM $CaCl_2$ and 0.05% Tween 20*

2.2. Protocols

Affinity purification

Column preparation

See section, **Working Solution** *for information on preparing solutions.*

1. Attach lower cap to bottom of column, then place column on rack or stand above desired collection tube.

2. Gently invert Anti-Protein C Affinity Matrix several times to thoroughly resuspend beads.

3. Pipette desired volume of slurry into column; suggested final settled bead volume is 0.5 to 1.0 ml.

4. Replace lower cap with a 23G needle.

5. Drain Column storage buffer into collection tube; adjust column flow rate to approximately 0.3 to 0.5 ml/minute.
   – If flow rate is faster, adjust with a smaller gauge needle at this time.
   Do not allow column to dry out.

6. Immediately add 10 bed volumes of Equilibration buffer, for example, 10 ml for a 1 ml column and allow buffer to drip through column.

7. Carefully remove needle and replace lower cap when buffer level reaches the top of the matrix bed.

Column loading

See section, **Working Solution** *for information on preparing solutions.*

1. Determine the amount of crude protein extract to be purified.
   – Optimal starting volume is 1 to 5 ml for a 1 ml column.
   – Optimal total protein concentration of extract is dependent upon expression levels of tagged protein. Use the following initial concentrations:

If starting lysate is	then total protein loaded/ml of matrix should be
bacterial,	1 – 3 mg
mammalian,	3 – 6 mg

   Column may become clogged if excessive total protein is loaded, or if insoluble materials have not been adequately removed from extract. Total protein loaded may be gradually increased with successive column runs, however, column should be able to flow at a rate of 0.1 ml/min or greater.

2. Add $CaCl_2$ from 1 M stock solution to protein extract in order to obtain a final concentration of 1 mM $CaCl_2$.
   Keep extract at +2 to +8°C.

3. Load protein extract on column and cap column tightly.

4. Invert column to mix.
   – Place column on end-over-end rocker at +2 to +8°C.
   – Incubate for a minimum of one hour, but not longer than overnight.

Column elution with EDTA

See section, **Working Solution** *for information on preparing solutions.*

1. Allow column matrix to settle for 10 minutes after mixing.
   – Remove top cap and replace lower cap with needle.

2. Collect lysate flow through in a clean collection tube.
   – Store this fraction at +2 to +8°C.

3. Wash column with a minimum of 10 bed volumes of Wash buffer at +15 to +25°C to remove nonspecifically bound protein.
   An OD_{280} reading performed at the end of this step will verify that the final wash fractions contain no protein and are close to baseline levels (Wash buffer alone).

4. Re-equilibrate column with 10 bed volumes of Equilibration buffer; allow buffer to drain through column.

5. Immediately replace needle with lower cap and add 1 ml of EDTA Elution buffer/ml of column matrix.
   – Incubate for 30 minutes at +15 to +25°C without shaking.

6. Replace needle and collect the first 1 ml elution fraction in a clean microfuge or equivalent collection tub.

7. Repeat Steps 5 and 6 four more times with a 5 minute incubation each time.
   Column may be eluted with one 5 ml bed volume of Elution buffer to save time, however, slightly lower yields of purified protein may result.

8. Read OD_{280} for each fraction and pool as desired.
   – In most instances, the first three fractions will contain the bulk of the eluted protein.

9. Keep samples at +2 to +8°C until analysis.

Column elution with peptide

See section, **Working Solution** *for information on preparing solutions.*

1. Allow column matrix to settle for 10 minutes after mixing.
   – Remove top cap and replace lower cap with needle.

2. Collect lysate flow through in a clean collection tube.
   – Store this fraction at +2 to +8°C.

3. Wash column with a minimum of 10 bed volumes of Wash buffer at +15 to +25°C to remove nonspecifically bound protein.
   An OD_{280} reading performed at the end of this step will verify that the final wash fractions contain no protein and are close to baseline levels (Wash buffer alone).

4. Equilibrate column with 10 bed volumes of Equilibration buffer; allow buffer to drain through column.

5. Immediately replace needle with lower cap and add 1 bed volume of Peptide elution buffer (peptide concentration of 0.5 mg/ml).
   – Incubate for 30 minutes at +15 to +25°C.
   – Gently mix the matrix once or twice during the incubation step to increase recovery.

6. Replace needle and collect the first 1 ml elution fraction in a clean microfuge or equivalent collection tub.

7. Repeat Steps 5 and 6 four more times with a 5 minute incubation each time.

8. Read OD_{280} for each fraction and pool as desired.
   – In most instances, the first three fractions will contain the bulk of the eluted protein.

9. Keep samples at +2 to +8°C until analysis.

Column reuse, regeneration, and storage

See section, **Working Solution** *for information on preparing solutions.*

For many EDTA elution protocols, if the protein to be purified is obtained from the same cell extract, it is not necessary to regenerate the column. Simply follow steps 1 to 3:

1. Wash with 10 bed volumes of Wash buffer.

2. Immediately equilibrate with 10 bed volumes of Equilibration buffer.

3. Store tightly capped at +2 to +8°C in 2 bed volumes of Column storage buffer.
   If different proteins are to be purified using the same column, the column must be stripped and regenerated between each purification. With peptide elution, it is always necessary to regenerate the column:

4. Strip column by running 20 to 30 bed volumes of Regeneration buffer through the column.

5. Immediately equilibrate column with 10 bed volumes of Equilibration buffer.

6. Store tightly capped at +2 to +8°C in 2 bed volumes of Column storage buffer.
   Other buffers and preservatives may be substituted, but have not been tested. Since calcium is a requirement for antibody binding with this system, avoid buffers containing phosphate to prevent precipitation.

Yield of purified protein

Yield of purified protein is dependent upon expression levels within crude extract. Typical results from a 1 ml column range from 2 to 10 nmol of purified protein. Using a whole-cell bacterial extract, which expressed Protein C-tagged β-gal, the column capacity was determined to be approximately 10.5 nmolprotein/ml resin. Average recovery from seven successive purifications performed on the same column with EDTA elution was 90% with no regeneration, and 93% with regeneration.

Number of times a column may be reused

Poured columns containing the affinity matrix may be used 7 to 10 times before a drop in yield of purified protein is observed. If a drop in yield is observed prior to the tenth column (use and regeneration has not been performed), stripping the column may improve the yield.

Immunoprecipitation

See section, **Working Solution** *for information on preparing solutions.*

1. Pipette 50 to 100 µl of resuspended matrix into each microcentrifuge tube to be used for immunoprecipitation.
   Use wide-bore pipette tips.

2. Pellet matrix with a brief pulse in the microcentrifuge.
   – Carefully remove supernatant using a fine-bore pipette tip, then resuspend matrix in 0.5 to 1 ml cold Cell lysis buffer.

3. Repeat Step 2, 1 to 2 more times.
   The affinity matrix may be used directly without pre-rinsing, however, occasional Ig bands may be observed during western blotting.

4. Add 0.5 to 1.0 ml of cold crude protein extract to final matrix pellet.

5. Incubate samples at +2 to +8°C on a rocker for 1 hour to overnight.
   Longer incubations increase binding to the affinity matrix.

6. Pellet matrix at full speed in a microcentrifuge for 30 seconds.
   – Carefully remove supernatant and rinse twice with 1 ml of cold Cell lysis buffer.

7. Perform final wash with 1 ml of Wash buffer, removing all residual buffer from pelleted matrix.

8. Use one of the following methods to elute tagged protein:

Method	Step
EDTA elution	Add 1 to 2 matrix volumes (50 to 200 µl) of EDTA elution buffer. – Rock at +2 to +8°C for 15 to 30 minutes. – Pellet matrix, saving supernatant. – Dilute supernatant in electrophoresis sample buffer or analyze as desired.
Direct elution into electrophoresis sample buffer	Add 1 to 2 matrix volumes of sample buffer and boil for 5 minutes. – Pellet matrix before gel electrophoresis.

Western blotting

See section, **Working Solution** *for information on preparing solutions.*

1. Perform gel electrophoresis according to standard protocols.
   – Wet PVDF membrane in 100% methanol and equilibrate the membrane in Transfer buffer.
   – Perform western transfer to the PVDF membrane.

2. After transfer, block the membrane for 1 hour at +15 to +25°C in Blocking buffer with gentle shaking.
   10 ml of Blocking buffer covers a 10 cm × 10 cm PVDF membrane.

3. Combine 10 ml Blocking buffer with 10 ml TBS-Ca^{2}+ solution.

4. Prepare working-strength Anti-Protein C (final antibody concentration, 0.1 to 1 µg/ml) by diluting Anti-Protein C stock solution with the solution prepared in Step 3.

5. Incubate the blocked membrane with working-strength Anti-Protein C for 1 hour at +15 to +25°C with gentle shaking.

6. Wash the membrane three times, 5 minutes per wash with 10 ml TBST-Ca^{2}+ solution.

7. Prepare 10 ml working-strength anti-mouse IgG (H+L)-POD by diluting the secondary antibody 1:4,000 with the solution prepared in Step 3.
   – Add the secondary antibody and incubate the membrane for 30 minutes at +15 to +25°C with gentle shaking.

8. Wash the membrane three times, 5 minutes per wash with 10 ml TBST-Ca^{2}+ solution.

9. Prepare Lumi-Light Western Blot Substrate (POD) according to the directions in the Instructions for Use.
   – Incubate membrane in this solution for 5 minutes.

10. Drain excess detection solution from membrane and wrap in plastic wrap.
    – Expose the membrane to X-ray film for 60 seconds according to the method provided with the substrate.
    Substrate development and X-ray film exposure conditions may vary for each experiment.

2.3. Parameters

Specificity

Anti-Protein C recognizes the 12-amino acid sequence EDQVDPRLIDGK, which encodes residues 6 through 17 of the heavy chain of Protein C. In the presence of Ca^{2+}, the antibody binds with high affinity and specificity to this sequence in native human protein C or in proteins tagged with this epitope. This efficient binding within the recombinant fusion protein occurs regardless of the site of incorporation of the epitope tag (N terminus, C terminus, or within the reading frame).

3. Results

Western blot (Fig. 1) and silver-stained gel (Fig. 2) demonstrating purification of Protein C-tagged β-galactosidase from a crude bacterial extract from both the N- and C-terminal positions. A 0.5 ml Anti-Protein C Affinity Matrix column was used to purify 3 mg of total protein.

Fig. 1: Western blot from crude bacterial extract.
Lane 1: Crude bacterial extract
Lane 2: Column flow through
Lane 3: Purified protein

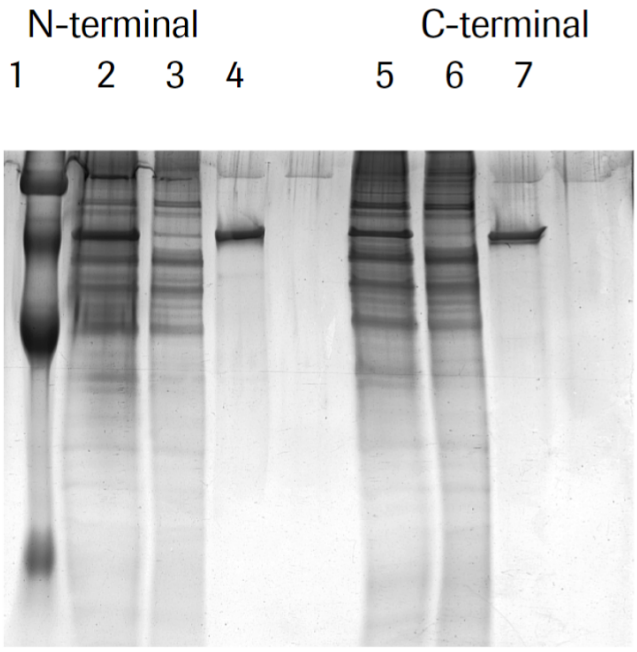

Fig. 2: Silver-stained gel from crude bacterial extract.
Lane 1: Molecular Weight Marker
Lanes 2 and 5: Crude bacterial extract
Lanes 3 and 6: Column flow through
Lanes 4 and 7: Purified protein

4. Troubleshooting

Observation	Possible cause	Recommendation
Little or no protein C-tagged protein is eluted.	Tagged protein is degraded.	Include protease inhibitors and perform purification at +2 to +8°C.
	Tagged protein not fully eluted.	If working at <+37°C, increase temperature, time, and/or number of elutions. Try batch mixing of peptide solution with matrix.
	Tagged protein expression is absent.	Check for expression of protein in crude extract by western blot or biochemical assay.
	Tagged protein expression is very low.	Load larger volume of extract. Run column several times, pool, and concentrate final eluates.
Large quantities of tagged protein remain in the flow through sample.	Column is overloaded.	Decrease amount of loaded protein extract.
	Column not regenerated after use.	Regenerate column.
Column flow stops.	Column is overloaded.	Decrease amount of loaded protein extract.
	Starting extract contains insoluble materials.	Preclear starting extract by high-speed centrifugation or filtration.
	Air bubble in needle.	Replace needle or place gentle pressure on column by briefly covering top of column with gloved hand.
Tagged protein appears degraded; a smear or multiple lower molecular weight bands on western blot.	Protease activity during procedure.	Increase protease inhibitors in protein extract sample.
		Perform all steps at +2 to +8°C.
Detection of Ig heavy or light chain on western blot following immunoprecipitation	Small quantities of heavy or light chain will accumulate over time in the matrix supernatant. This does not affect the performance (capacity) of the matrix before its expiration date.	Pre-rinse affinity matrix before using for immunoprecipitation.

5. Additional Information on this Product

5.1. Quality Control

For lot-specific certificates of analysis, see section, **Contact and Support**.

6. Supplementary Information

6.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

6.2. Changes to previous version

Layout changes.
Editorial changes.

6.3. Ordering Information

Product	Pack Size	Cat. No.
Reagents, kits		
Lumi-Film Chemiluminescent Detection Film	100 films, 7.1 x 9.4 inches, 18 x 24 cm, *Not available in US*	11 666 916 001
	100 films, 8 x 10 inches, 20.3 x 25.4 cm	11 666 657 001
PVDF Western Blotting Membranes	1 roll, 30 cm x 3.00 m	03 010 040 001
Tween 20	50 ml, 5 x 10 ml	11 332 465 001
Tris hydrochloride	500 g	10 812 846 001
Tris base	1 kg, *Not available in US*	10 708 976 001
	1 kg	03 118 142 001
	5 kg	11 814 273 001
Western Blocking Reagent, Solution	100 ml, 10 blots, 100 cm^2	11 921 673 001
	6 x 100 ml, 60 blots, 100 cm^2	11 921 681 001
Lumi-Light Western Blotting Substrate	1 kit, 4,000 cm^2 membrane, 400 blots with 10 x 10 cm	12 015 200 001
cOmplete, EDTA-free	20 tablets, for 50 ml each	04 693 132 001
	20 tablets in a glass vial, for 50 ml each	11 873 580 001
	3 x 20 tablets in glass vials, for 50 ml each	05 056 489 001
Nonidet P-40 Substitute	50 ml, 5 x 10 ml	11 332 473 001

6.4. Trademarks

All product names and trademarks are the property of their respective owners.

6.5. License Disclaimer

For patent license limitations for individual products please refer to:
List of biochemical reagent products.

6.6. Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

6.7. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

6.8. Contact and Support

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support Site**.

To call, write, fax, or email us, visit **sigma-aldrich.com**, and select your home country. Country-specific contact information will be displayed.

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