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Clusterin-VLDLR-ed Binding Assay

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Andreas Bracher[1], Patricia Yuste-Checa[1], F Ulrich Hartl[1]

[1]Department of Cellular Biochemistry, Max Planck Institute of Biochemistry, Martinsried, Germany

Patricia Yuste-Checa
MPI Biochemistry

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We use this protocol and it's working

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Abstract

This protocol details how to show the interaction between Clusterin (Clu) and the C-tagged ecto-domain of Very Low Density Lipoprotein Receptor (VLDLR) in vitro by immunoprecipitation.

Materials

- C-tag wash buffer

	A	B
	Tris-HCl pH 7.4	20 mM
	NaCl	100 mM
	$CaCl_2$	2 mM

- C-tag elution buffer

	A	B
	Tris-HCl pH 7.0	20 mM
	$MgCl_2$	2 M
	$CaCl_2$	2 mM

- CaptureSelect C-tag affinity resin (Thermo Fisher Scientific, 2943072005)
- Empty spin columns for standard 1.5 ml reaction tubes (Mo Bi Tec, M1002S)
- Clusterin (see protocol “Clusterin purification from HEK293E cells”, dx.doi.org/10.17504/protocols.io.bvvkn64w).
- RAP (see protocol “Purification of recombinant Low Density Lipoprotein Receptor Related Protein Associated Protein 1 (LRPAP1, RAP) from Escherichia coli”, dx.doi.org/10.17504/protocols.io.rm7vzxpb2gx1/v1)
- VLDLR-ed (VLDLR(28-797)Δ(751-779)-C-tag, derived from Uniprot isoform sequence P98155-2) (see protocol “Very low-density lipoprotein receptor (VLDLR)-C-tag purification from HEK293E cells”, dx.doi.org/10.17504/protocols.io.j8nlkoqw1v5r/v1)
- NuPAGE 4–12% Bis-Tris SDS gels (Thermo Fisher Scientific)
- NuPAGE MES SDS running buffer (Thermo Fisher Scientific)
- 2x SDS-PAGE sample buffer

	A	B
	Tris-HCl pH 6.8	100 mM
	Dithiothreitol (DTT)	0.2 M
	Sodium dodecyl sulfate (SDS)	4%
	l-1 bromophenol blue	2 g
	Glycerol	20%

Note

NOTE: Prepare by mixing 1.54 g DTT, 0.10 g bromophenol blue, 10 ml glycerol, 5 ml of 1 M Tris-HCl pH 6.8 stock and 20 ml of 10% (w/v) SDS stock. Top up with Milli-Q de-ionized water to 50 ml total volume. Make 1-ml aliquots and store at -20 °C.

- Pre-stained molecular weight SDS-PAGE marker (PageRuler Prestained Protein Ladder, 10 to 180 kDa, ThermoFisher)
- Select Western blot transfer stacks nitrocellulose (Invitrogen)
- Tris-buffered saline containing 0.05% Tween 20 (0.05% TBS-T)

TBS

	A	B
	Tris-HCl pH 7.5	10 mM
	NaCl	150 mM

Note

NOTE: Prepare freshly from 10x TBS-T stock, 100 mM Tris-HCl pH 7.5, 1500 mM NaCl, 0.5% Tween 20.

- Stripping buffer

	A	B
	Glycine pH 2.0	20 mM
	SDS	2%

- PBS-T (1x PBS with 0.1% Tween 20)
- 3% bovine serum albumin (BSA) (Merck) in TBS-T. Store at 4 °C.
- Mouse monoclonal Clu-α antibody (Santa Cruz Biotechnology, sc-5289, 1/1000 dilution)
- Conjugated goat-anti mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) (Merck, A4416)
- CaptureSelect biotin anti-C-tag conjugate (Thermo Fisher Scientific, 7103252100, 1/2000 dilution) Streptavidin-HRP (Pierce, 21130, 1/10000 dilution)
- Immobilon Forte Western HRP substrate (Merck)

Instruments

- Microcentrifuge for 1.5 ml reaction tubes (Eppendorf)
- Vortex mixer (Vortex-Genie 2, Scientific Industries)
- Thermo mixer (Eppendorf Thermomixer Comfort)

- Semi-dry blotting apparatus (Power Blotter XL, Invitrogen)
- Luminescence gel documentation system (ImageQuant LAS 4000 mini, General Electric)

CaptureSelect™ C-tagXL Affinity Matrix **Thermo Fisher Catalog #**2943072005

Clusterin-α Antibody (B-5) **Santa Cruz Biotechnology Catalog #**sc-5289

Anti-Mouse IgG (whole molecule)–Peroxidase antibody produced in goat **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A4416

CaptureSelect™ Biotin Anti-C-tag Conjugate **Thermo Fisher Catalog #**7103252100

Preparatory Work

1 Completely resuspend C-tag affinity resin in storage buffer by gentle shaking.

2 Transfer approximately 50 µL C-tag affinity resin into each empty spin columns.

Note

Consider the dilution factor in the suspension, e.g. if the total volume is twice the bead volume, transfer 100 µl suspension. The nozzle of the pipette tip might be too narrow for the gel beads. Cut off the tip with scissors for easy transfer.

3 Let the storage buffer pass through by spinning at 2000 rpm, 00:02:00 in a microcentrifuge. 2m

4 Equilibrate columns with 250 µL C-tag wash buffer.

5 Remove residual C-tag wash buffer by spinning at 2000 rpm, 00:02:00 in a microcentrifuge. 2m

6 Plug the column outlets.

Complex Formation and Column Binding

7 Use following concentrations (µM) for your samples:

	A	B	C	D
	Sample	**Clu**	**VLDLR-ed**	**RAP**
	Clu-Bkg	5		
	Clu-VLDLR	5	5	
	RAP-VLDLR (RV)		5	5

	A	B	C	D
	RAP-Bkg (R)			5

8 Calculate the necessary volumes of the Clusterin, VLDLR-ed and RAP stock solutions for 100 µL total volume. The volume of C-tag affinity resin is 50 µL. Fill up to 100 µL total volume with C-tag wash buffer.

9 Resuspend C-tag affinity resin by gentle vortexing.

10 Remove from each column 10 µL sample (“Input”) for SDS-PAGE.

11 Incubate for 02:00:00 at 25 °C with gentle shaking (750 rpm) in thermomixer. 2h

12 Remove column plugs. Place fresh 1.5 ml collection tubes under the columns. Label tubes.

13 Harvest unbound residual C-tag wash buffer by spinning at 2000 rpm, 00:02:00 in a microcentrifuge. 2m

14 Replace collection tubes. Label tubes.

15 Wash four times with 100 µL C-tag wash buffer. Each time resuspend resin, followed by centrifugation. Collect wash solution.

16 Replace collection tubes. Label tubes.

17 Elute bound proteins three times 50 µL C-tag elution buffer. Each time resuspend resin, followed by centrifugation. Collect eluate.

SDS-PAGE

18 6m

- Prepare SDS-PAGE samples by mixing sample and 2x SDS-PAGE sample buffer at 1:1 ratio.
- Then heat SDS-PAGE samples to 95 °C for 00:05:00 in metal heating block, followed by cooling in a cold metal block.
- Centrifugation for 14000 rpm, 00:01:00 in microcentrifuge.

Note

The DTT in the 2x SDS-PAGE sample buffer quickly perishes by air oxidation. Keep chilled on ice! To avoid Clusterin gel artifacts by incomplete reduction of its five disulfide bonds, do not re-use 2x SDS-PAGE sample buffer, once it was thawed.

19 Assemble electrophoresis apparatus with NuPAGE 4-12% Bis-Tris SDS gels. Fill inner and outer chamber with NuPAGE MES SDS running buffer (1x).

20 Load NuPAGE 4-12% Bis-Tris SDS gels with input samples (5 µL), unbound (5 µL), wash (20 µL) and elution (7.5 µL) SDS-PAGE samples. Use pre-stained SDS-PAGE molecular weight marker (5 µL) as a standard.

Note

NOTE: The unequal loading volumes result in equal dilution on the gel.

21 Run electrophoresis for 00:45:00 at a constant voltage of 200 V. 45m

22 Analyze gel with RAP-containing samples by Coomassie-blue staining.

Note

This will show the outcome of the binding experiments with RAP as a positive control.

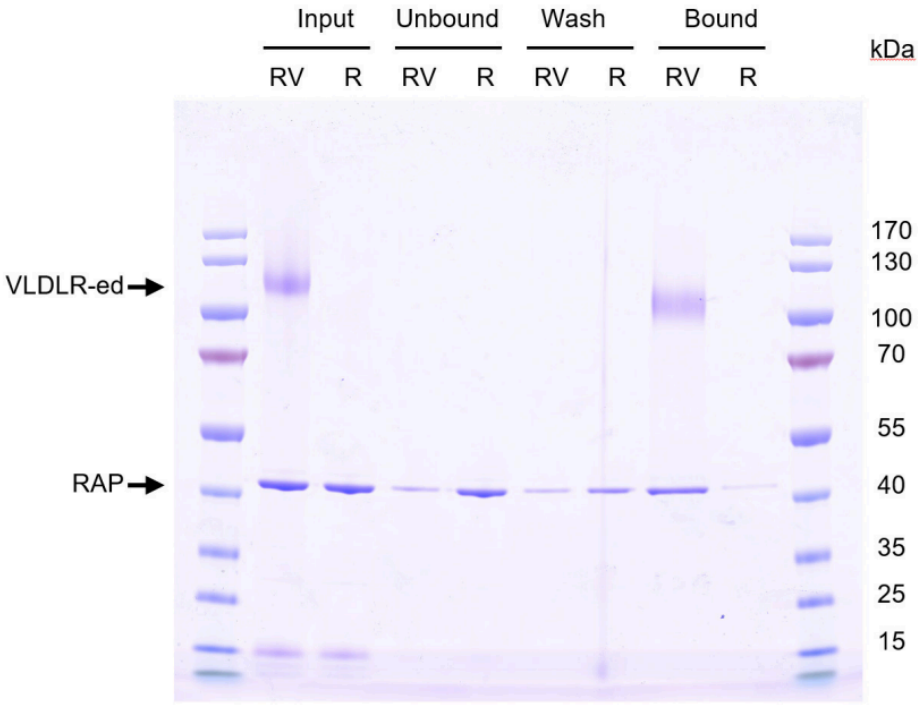

Fig. 1 Coomassie-stained SDS-PAGE showing the binding of RAP to immobilized C-tagged VLDLR-ed (VR). RAP alone (R) shows only residual binding to the C-tag affinity resin. The bands for VLDLR-ed appear fuzzy probably because of heterogeneity of attached O- and Nglycans.

Western Blot Transfer and Anti-Clusterin Immunostaining

23
- Carefully pry open the plastic casing of the NuPAGE 4–12% Bis-Tris SDS gel by using a so-called gel knife (Invitrogen) or similar.
- Remove the loading pockets with a scalpel while the gel is still attached to the plastic.

24 Place the gel into a staining dish and briefly wet with de-ionized water.

25 For electro-blotting, follow the steps in the instructions to the Power Blotter system (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017051_PBSelectStacks_QR.pdf).

26 After disassembly of the blotting stack, attach the nitrocellulose membrane to the inner wall of a tubular vessel and wash three times with 10 mL TBS-T while tumbling on a roller mixer.

Note

The protein bands have to face inside. The complete surface of this membrane face has to be accessible to the buffer. Do not touch membrane with bare fingers.

27 Block nitrocellulose membrane by incubation with 10 mL 3% BSA in TBS-T for 01:00:00 at Room temperature using a tubular vessel and a roller mixer. 1h

28 Wash the nitrocellulose membrane three times with 10 mL TBS-T.

29 Incubate with mouse monoclonal Clu-α antibody, 1/1000 diluted with 10 mL TBS-T, Overnight in the cold room using a tubular vessel and a roller mixer.

30 Wash the nitrocellulose membrane three times with 10 mL TBS-T.

31 Incubate with conjugated goat-anti mouse IgG-HRP, 1/2000 diluted with 10 mL TBS-T for 01:00:00 at Room temperature using a tubular vessel and a roller mixer. 1h

32 Wash the nitrocellulose membrane three times with 10 mL TBS-T.

33 Transfer the nitrocellulose membrane onto a glass plate.

34 Using the gel documentation system, take a photo in epi-illumination mode.

35 Cover the nitrocellulose membrane with 3 mL Immobilon Forte Western HRP substrate.

Note

Do not move the glass plate.

36 Record 10 s-incremental exposures in luminescence mode until the first pixels start to saturate.

Membrane Stripping and Anti-C-tag Immunodetection

37

Note

This is an optional control to make sure that nothing went wrong in the initial steps (steps 8-17).

Wash the nitrocellulose membrane three times with 10 mL water using a tubular vessel and a roller mixer.

38 To strip the antibodies from the nitrocellulose membrane,

38.1 Incubate the nitrocellulose membrane twice with 10 mL Stripping buffer for 00:05:00 using a tubular vessel. (1/2) 5m

38.2 Incubate the nitrocellulose membrane twice with 10 mL Stripping buffer for 00:05:00 using a tubular vessel and a roller mixer. (2/2) 5m

39 Wash the nitrocellulose membrane three times with 10 mL water.

40 Wash the nitrocellulose membrane three times with 10 mL PBS-T.

41 Incubate with Biotin anti-C-tag (Thermo), 1/4000 diluted with 1% skimmed milk powder, in PBS-T, for 02:00:00 at Room temperature using a tubular vessel and a roller mixer. 2h

42 Wash the nitrocellulose membrane three times with 10 mL PBS-T.

43 Incubate with Streptavidin-HRP (Pierce), 1/10000 diluted with 1% BSA in PBS, for 01:00:00 at Room temperature using a tubular vessel and a roller mixer.

1h

44 Wash the nitrocellulose membrane three times with 10 mL PBS-T.

45 Detect marker bands and luminescence signal as in steps 33-36.

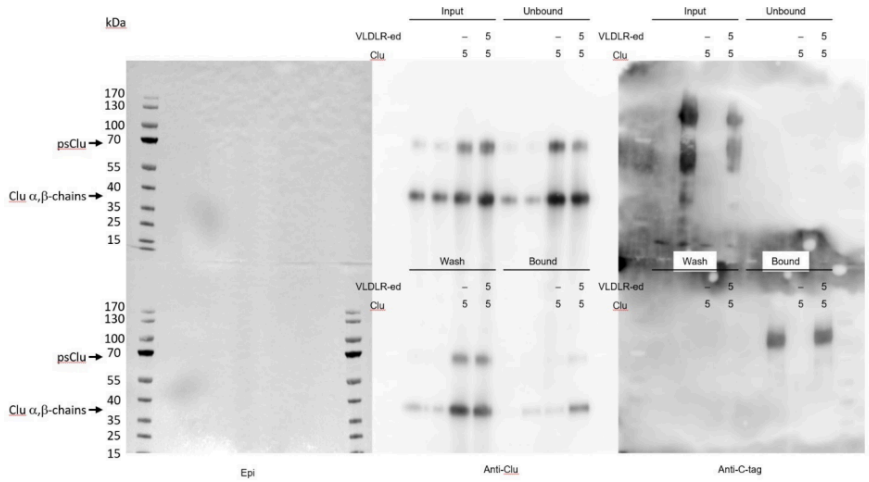

Fig. 2 Side-by-side comparison of marker bands (epi-illumination), anti-Clusterin (Clu) and antiC-tag (C-tagged VLDLR-ed) immunoblots. Protein from two gels (top and bottom) was transferred onto one nitrocellulose membrane. The respective concentrations (µM) of VLDLR-ed and Clusterin are indicated above the lanes. The additional lanes show experiments at a different Clu:VLDLR ratio (1:10).