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ATG9A-vesicle isolation protocol

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We use this protocol and it's working

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Abstract

This protocol details about the ATG9-vesicle isolation.

Materials

Sodium-Orthovanadate (Applichem, prepared as [M] 100 millimolar (mM) in MQ, frozen)

b-Glycerophosphate (Sigma, prepared as [M] 1 Molarity (M) in MQ, frozen)

NaF (Sigma, prepared as [M] 500 millimolar (mM) in MQ, frozen)

Complete EDTA free protease inhibitor tablets, Sigma 5056489001 (prepared as 50x stock 1 tbl in 1 ml H2O)

Anti-FLAG M2 Affinity Gel from Sigma A2220-1ml

D(+)-Saccharose (sucrose) from Roth 4661.1

FLAG Peptide from Sigma F32920-4MG

Dissolved at 4 mg /ml in SEC buffer ([M] 25 millimolar (mM) NaCl, [M] 1 millimolar (mM) DTT), aliquoted, kept at -20 °C

3 mL Luer Lock HENKE-JECT syringe

Braun Sterican 26 G x 1“ Gr. 18 needle

MilliQ water (filtered additionally through a 0.2µm membrane)

All buffers used were filtered prior to use

0.2 µm celluloseacetate syringe filter from Chromafil CA-20/25 (S)

	A	B	C	D	E	F
			Vesicle Isolation Buffer		Elution Base Buffer	
	Reagent	Stock conc	Final conc	Vol for 20ml	Final Conc	Vol for 20ml
	HEPES pH7.5	1M	20mM	400µl	20mM	400µl
	NaCl	5M	150mM	600µl	150mM	600µl
	Sucrose	1M	250mM	5ml	-	-
	Complete EDTA free protease inhibitors (PI)	50x	1x	400µl	1x	400µl
	Beta-Glycerophosphate	1M	20mM	400µl	20mM	400µl
	Sodium-Orhovanadate	100mM	1mM	200µl	1mM	200µl
	NaF	500mM	1mM	40µl	1mM	40µl
	EDTA pH8.0	0.5M	1mM	40µl	-	-
	In MilliQ H2O					
	Sterile filtered, precooled					

Before start

Hap1 cells were previously CRISPR engineered in our lab to contain ATG9A endogenously tagged on the C-terminus with mEGFP-3C-FLAG.

Preparation of starting material (Hap1 ATG9A-mEGFP-3C-FLAG cell pellets)

1 Grow and expand cells in IMDM medium supplied with 10% FCS and Pen/Strep until the required number of cells is reached. 2w
Prep size: For one preparation of vesicles the required amount is 100 x 10^6 to 150 x 10^6 cells which is equivalent to 4 - 5 x 15 cm dishes of confluent cells.
Detach cells with trypsin, count and aliquot to prep size (see above), wash with cold PBS, spin down 200 rcf, 4°C , remove supernatant, flash freeze the cell pellets and keep at -80 °C until use.

Day1: Cell lysis and binding to Flag beads 1h

2 **Equilibrate Flag beads**
Always pellet the beads with 4000 rcf, 4°C cooled 5415R centrifuge for 3'.

2.1 Take 70 µL of FLAG bead slurry per prep.

2.2 Wash 3x with MilliQ water

2.3 Wash 3x with Vesicle Isolation Buffer.

2.4 Prepare a 1 : 1 slurry in **Vesicle isolation Buffer**

3 **Cell lysis**
Always work On ice
">> sample name" means: take a sample for Western Blot e.g. 10 µl sample + 2 µl 6xProtein loading dye

3.1 Thaw cells On ice

3.2 **Resuspend** the pellet in 1665 µL of **Vesicle Isolation Buffer** and transfer to a fresh 2ml Eppi.

3.3 Incubate On ice for 00:20:00 20m

3.4 Lyse the cells by pushing them **30 times (up and down) through a 26 x g needle** 10m

3.5 Let them chill for 00:10:00 On ice 10m

3.6 Repeat the lysis step **(push 30 times up and down through a 26 x g needle)** 10m

3.7 Pellet the cell debris and nuclei (200 rcf, 4°C, 00:06:00) 6m

3.8 Transfer the supernatant to a fresh 2 ml Eppi (>> Sample Supernatant)

4 Add 70 µL of equilibrated **FLAG beads** (slurry). 10m
Incubate Overnight at 4 °C (on the roller in the cold room in a 50ml Falcon tube)

Day2 Elution of ATG9A vesicles 3h 15m

5 Pellet the beads (in a 5415R centrifuge) at 200 rcf, 00:03:00 and remove the supernatant (>> Sample Unbound). 3m

6 Resuspend the beads in 1 mL of **Vesicle Isolation Buffer** and transfer to a fresh 2ml Eppi (+ 665 µL **Vesicle Isolation Buffer** already pre-pipetted in the Eppi).

7 Incubate 00:01:00 rolling at 4 °C or on ice with occasionally inverting the Eppi carefully 1m

8 Pellet the beads at 2000 rcf, 00:03:00 and remove the supernatant 3m

9 Resuspend the beads in 1 mL of Vesicle Isolation Buffer and transfer into a fresh 2 ml LowBind tube

9.1 Add 665 µL of **Elution Base Buffer** very slowly to the tube (decreases the sucrose concentration;
now be even more careful than before!)

10 Incubate 00:01:00 rolling at 4 °C or on ice with occasionally inverting the tube carefully
1m

11 Pellet the beads at 2000 rcf, 00:03:00 and remove the supernatant
3m

12 Resuspend the beads in 1 mL of **Elution Base Buffer** and transfer into a fresh 2 ml LowBind tube 665 µL **Elution Base Buffer** already in pre-pipetted in the tube).

13 Incubate 00:01:00 rolling at 4 °C or on ice with occasionally inverting the tube carefully
1m

14 Pellet the beads at 2000 rcf, 00:03:00 and remove the supernatant.
3m

15 Resuspend the beads in 666 µL of **Elution Base Buffer** and transfer to a fresh 1.5 ml LowBind tube (>> Sample Elution Input).

16 Add 16.65 µL of 4 mg /ml **FLAG peptide**

17 Incubate for 02:45:00 at 4°C rolling
2h 45m

18 Pellet the beads at 2000 rcf, 00:03:00 and transfer the supernatant to a fresh LowBind tube (>> Sample Elution)
This is your vesicle prep! Handle with care.
3m

19 Resuspend the beads again in 666 µL of **Elution Base Buffer** (>>Sample Beads after Elution)