May 07, 2024

Generation of stable LysoTag expressing cell lines and LysoTag immunoprecipitation of lysosomes

DOI
dx.doi.org/10.17504/protocols.io.261gedz2ov47/v1

Daniel Saarela[1,2], Dario Alessi[1,2]

[1]Aligning Science Across Parkinson's; [2]MRC-PPU at The University of Dundee

ASAP Collaborative Rese…

Francesca Tonelli
MRC-PPU at The University of Dundee

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.261gedz2ov47/v1

Protocol Citation: Daniel Saarela, Dario Alessi 2024. Generation of stable LysoTag expressing cell lines and LysoTag immunoprecipitation of lysosomes. **protocols.io https://dx.doi.org/10.17504/protocols.io.261gedz2ov47/v1**

Protocol status: Working
We use this protocol and it's working

Created: February 14, 2024

Last Modified: May 07, 2024

Protocol Integer ID: 95330

Keywords: ASAPCRN, LysoTag, lysosomes

Funders Acknowledgement:
Aligning Science Across Parkinson's
Grant ID: ASAP-000463

Disclaimer

Abstract

Molecular homeostasis in cells is regulated in part by protein degradation, which is facilitated by the proteasome and lysosomal proteolysis. Lysosomes are membrane bound organelles involved in the turnover of proteins, metabolites and lipids. Recent literature implicates lysosomal dysfunction to be a feature of many a disease, including neurodegenerative diseases. Focused investigation of lysosomal content (proteome/lipidome/metabolome) in disease states could lead to the discovery of novel therapeutics and disease mechanisms.

Here we describe how to produce stable LysoTag expressing cell lines and how to perform rapid isolation of lysosomes in cultured cells with immunoprecipitation of the LysoTag using HA-coupled magnetic beads. The immunoprecipitation protocol is very fast, less than 15min from start of the incubation with the beads. The protocol can be used to immunoprecipitate lysosomes from commonly cultured cells such as mouse embryonic fibroblast, HEK293 and A549 cells expressing the LysoTag.

Attachments

1014-2621.pdf
65KB

Materials

Cell lines :

Invitrogen™ 293FT Cell Line **Invitrogen - Thermo Fisher Catalog #**R70007

Human Embryonic Kidney (HEK293) Cells **ATCC Catalog #**CRL-1573

Plasmids :

- pLJC5 TMEM192 3XHA (DU68356 available at MRCPPU depository at MRCPPUreagents@dundee.ac.uk). This is the LysoiTag expression construct
- pLJC5-KOZAK-3HA-Empty (DU70022 available at MRCPPU depository at MRCPPUreagents@dundee.ac.uk). This is the MockTag expression construct
- pVSVG. Lentivirus envelope plasmid. Lenti-X HTX Packaging system (Clonetech. Catalog# 631247).
- pGag/Pol. Lentivirus Gag/Pol plasmid. Lenti-X HTX Packaging system (Clonetech. Catalog# 631247).
- QIAGEN HiSpeed® Plasmid Maxi kit [Lot# 166034460]

Media and Reagents:

Growth Media:

	A	B
	Dulbecco's Modified Eagle's Medium (DMEM)	
	Foetal Bovine Serum (FBS)	10%
	L-Glutamine	1%
	PenicillinStreptomycin	1%

DMEM (Gibco™ #11960-085) **Gibco - Thermo Fischer Catalog #**11960085

Fetal Bovine Serum **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F7524

L-Glutamine (200mM) **Thermo Fisher Scientific Catalog #**25030024

Penicillin-Streptomycin **Gibco - Thermo Fischer Catalog #**15140122

Selection Media: Puromycin dihydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P9620

Transfection media: Opti-MEM (Reduced Serum Medium) **Thermo Fisher Scientific Catalog #**31985062

DPBS no calcium no magnesium **Gibco - Thermo Fisher Catalog #**14190169)

KPBS Buffer: Adjust to pH 7.25 with KOH. (Note On the day of use, add Roche cOmplete protease inhibitor cocktail tablet (REF# 11873580001) and Roche PhosSTOP tablet (REF# 04906837001)

KPBS Buffer:

	A	B
	KCL	136 mM
	KH2PO4	10 mM

- cOmplete™ EDTA-free Protease Inhibitor Cocktail **Roche Catalog #**11873580001
- Roche PhosSTOP™ **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**4906837001

PEI MAX® - Transfection Grade Linear Polyethylenimine Hydrochloride (MW 40000) **Polysciences, Inc. Catalog #**24765-1

- Polybrene Infection / Transfection Reagent **Merck Millipore (EMD Millipore) Catalog #**TR-1003-G

Equipment:

- Isobiotec Cell-Breaker, isobiotec Vertriebs UG

Equipment

The Belly Dancer Shaker (Orbiter)	NAME
The Belly Dancer®	BRAND
BDRAA115S	SKU
https://www.ibisci.com/products/belly-dancer-shaker	LINK

- DynaMag™- Spin Magnet **Thermo Fisher Catalog #**12320D
- Incubator with FPI-sensor system and display controller MB1 (BINDER GmbH. Model: CB150. Power Output: 1.40kW, 230V, 6.1 Amp). This incubator has CO_2 and O_2 control.

Equipment

Microcentrifuges, Micro Star 17R (VWR #521-1647)	NAME
Microcentrifuges	TYPE
Micro Star 17R	BRAND
521-1647	SKU
https://in.vwr.com/store/product/8306728/microcentrifuges-ventilated-refrigerated-micro-star-17-17r	LINK

- Stripetter/stripette gun and stripettes
- Set of gilson pipettes P10, P200, P1000

Consumables:

- Pierce™ Anti-HA Magnetic Beads **Thermo Fisher Catalog #**88837
- Nunc™ Cell Culture/Petri Dishes, 56.7cm2, Nunclon Delta treated, lid, vent **Thermo Fisher Catalog #**172931
- Nunc™ Cell Culture/Petri Dishes, 145 cm2, Nunclon Delta treated,lid, vent **Thermo Fisher Catalog #**168381
- SafeSeal reaction tube 1.5 ml PP PCR Performance Tested Low protein-binding **Sarstedt Catalog #**72.706.600
- 15 mL conical centrifuge tube **greiner bio-one Catalog #**188271
- 50 mL conical centrifuge tube **greiner bio-one Catalog #**227261
- PIPETTE TIPS 100- 1000 µL BLUE SUITABLE FOR EPPENDORF STERILE 60 PIECES PER RACK **greiner bio-one Catalog #**686271
- PIPETTE TIP 10 - 100 µL SUITABLE FOR EPPENDORF 96 PIECES / ST RACK **greiner bio-one Catalog #**685261
- Syringe Filter **Sartorius Catalog #**ST16537-Q
- Fisherbrand™ Cell Lifters **Thermo Fisher Scientific Catalog #**08-100-240
- Becton Dickinson Disposable needles 21G x 1 1/2 inch **Becton Dickinson (BD) Catalog #**304432
- Terumo® Syringe 3-part Syringe **Terumo Catalog #**MDSS01SE
- Syringes (10ml) (Medicina. REF# IVS10. LOT# 19111004)

Packaging LysoTag and HA-Empty (Mock) construct

2d 0h 25m

1

Note

This is done under sterile condition in a category 2 biological safety cabinet.

2 Grow one 10cm Petri dish of HEK293FT cells to 60% confluency per transfection (2)

3 Prepare a transfection mix to generate LysoTag expressing lentivirus in 1.5ml Eppendorf tube containing:

a. 3.8 µg pGag/Pol plasmid

b. 2.2 µg pVSVG plasmid

c. 6 µg pLJC5 TMEM192 3XHA plasmid

d. 300 µL OptiMem

Note

We purify plasmids using a QIAGEN HiSpeed® Plasmid Maxi kit [Lot# 166034460] following manufactures protocols and ensure sterile reagents are used and mixtures prepared in tissue culture hood to avoid contamination.

4 Prepare a transfection mix to generate MockTag expressing lentivirus in 1.5ml Eppendorf tube containing:

a. 3.8 µg pGag/Pol plasmid

b. 2.2 µg pVSVG plasmid

c. 6 µg pLJC5 short kozak 3HA plasmid

d. 300 µL OptiMem

5 Prepare two PEI mixtures in 1.5ml Eppendorf tube (one per transfection mix).

5.1 Dissolve 20 µL 1 mg/ml PEI Max 40K in distilled water.

5.2 300 µL OptiMem

6 Incubate each mixture separately for 00:05:00 at Room temperature . 5m

7 Add the PEI mixture (Step 5) to the transfection mix (Steps 3 or 4) and repeat for the MockTag.

8 Mix by gently ting up and down and incubate for 00:20:00 at Room temperature . 20m

9 Add each mixture dropwise using a P1000 sterile pipette into a 10cm HEK293FT containing Petri dish per transfection.

10 Incubate cells at 37 °C for 24:00:00 . 1d

11 Replace the growth media with fresh Growth Media and incubate cells at 37 °C for further 24:00:00 . 1d

12 Collect the media that contains the lentivirus and pass through 0.45µm syringe filter. This is now the lentivirus infection media. This could be used immediately or snap frozen in liquid nitrogen and stored at -80 °C .

Infecting cells to stably express Lyso/MockTag 6d

13

Note

This is done under sterile condition in a category 2 biological safety cabinet.

Grow HEK293 cells in a 10cm Petri dish to 60% confluency.

14 Mix 5 mL of infection media (Step 12) with 5 mL of fresh growth media of your cells you are intending to infect in a 15ml Falcon tube.

15 Add Polybrene reagent (10 µg/ml) dissolved in MilliQ water) to the infection mix (Step 14) to a final concentration [M] 10 Mass Percent Polybrene.

16 Remove growth media from your HEK293 cells (Step 13).

17 Gently add the mix (Step 14) to the HEK293 cells (Step16).

Note

This method can be used to infect other cells as well (A549, MEF, HELA).

18 Incubate at 37 °C for 24:00:00 . 1d

19 Replace the infection media with fresh normal growth media and further incubate at 37 °C for 24:00:00 . 1d

20 Select for cells expressing your Lyso/Mock Tag by replacing the growth media with selection media for 24:00:00 . 1d

21 Remove and replenish the selection media every 24:00:00 for 72:00:00 . 3d

Note

Pay attention to the survival and confluency of your cells. There will be significant cell death observed and the plate is likely to only reach confluency in 48-72h.

22 Once the transfected cells reach confluency in transfection media, expand and/or freezestore cells for use for LysoTag-immunoprecipitation experiments.

Pre-clearing of anti-HA beads

2m

23

Note

This should be done on the day of immunoprecipitation experiment and On ice / 4 °C .

Pipette 100 µL anti-HA bead slurry into 1.5ml Eppendorf tube.

24 Place the tube containing the bead slurry onto a tube magnet for 00:00:30 . 30s

25 Remove supernatant and resuspend with 100 µL of ice cold KPBS off magnet, making sure to disperse clumps from the slurry.

26 Repeat steps 24 and 25, 3 more times.

26.1
- Place the tube containing the bead slurry onto a tube magnet for 00:00:30 . 30s
- Remove supernatant and resuspend with 100 µL of ice cold KPBS off magnet, making sure to disperse clumps from the slurry. (1/3)

26.2
- Place the tube containing the bead slurry onto a tube magnet for 00:00:30 . 30s
- Remove supernatant and resuspend with 100 µL of ice cold KPBS off magnet, making sure to disperse clumps from the slurry. (2/3)

26.3
- Place the tube containing the bead slurry onto a tube magnet for 00:00:30 . 30s
- Remove supernatant and resuspend with 100 µL of ice cold KPBS off magnet, making sure to disperse clumps from the slurry. (3/3)

27 Store On ice for later use.

Note

This amount of bead slurry can perform one Lyso/MockTag IP. Scale up volumes in the factor of 15cm Petri dishes of cells you are intending to use for your experiment.

Preparation of Isobiotec cell breaker

28 To prepare the Isobiotec cell-breaker, assemble it by inserting the ball inside the machine and screw the lids on tightly. Place on aluminium foil On ice and push 3 mL of KPBS through the machine to wash it. Carefully tap dry.

Note

- There will be residual KPBS left in the cell-breaker (approximately 200 µL), this is optimal.
- The ball size is determined by your cell type. We have found using 10 µm gap is optimal for HEK293 and A549 cells whereas for MEF cells 12 µm gap is preferred.

Homogenisation of Lyso/MockTag expressing HEK293 cells

4m

29

Note

Steps should be done separately for both LysoTag and MockTag expressing cells. Additionally, only process as many plates at a time as you have capability to process in a rapid manner. This will also depend on how many Isobiotec cell breaker you have access to.
Eg. If you have 2 Isobiotec cell breakers, only process 2 dishes for homogenisation at a time.

Grow cells to a confluency of 80-90% in 15cm Petri dishes.

30 Place cells on aluminium covered ice and remove media.

31 Add 5 mL ice cold PBS and swirl it to cover all of the plate.

32 Remove the PBS and add another 5 mL ice cold PBS and swirl the plate.

33 Remove the PBS and add 800 µL ice cold KPBS to the top of the plate.

34 Scrape off the cells in the KPBS with a cell lifter.

35 Transfer the cell/KPBS mixture to a 1.5ml Eppendorf tube using a P1000 pipette.

36 Pellet the cells at 1500 x g , 4 °C , 00:02:00 . 2m

37 Discard the supernatant carefully to not disturb the pellet.

38 Resuspend the pellet in 800 µL ice cold KPBS using a P1000 pipette.

39 Take 50 µL of the cell suspension aside as your whole cell sample (WC)

> **Note**
>
> Pellet the WC sample at 1500 x g , 4 °C , 00:02:00 , aspirate the supernatant
> and place On ice .

40 Using a 1ml syringe and 21 gauge needle, aspirate the cell suspension(Step 38) into the syringe and discard the needle.

41 Transfer the cell suspension into a KPBS rinsed, ice-cold Isobiotec cell-breaker with gapsize of 10 µm. Homogenise the cells with 15 passes through the cell breaker using two 1ml syringes.

42 Collect the homogenate from the cell breaker into a fresh 1.5ml Eppendorf.

Note

To collect as much as possible from the cell-breaker, push air into the cellbreaker using a syringe and collect using another.

43 Pellet at 1000 x g for 00:02:00 at 4 °C . 2m

Note

Your supernatant now contains the cytoplasm and organelles whilst the pellet contains non-homogenised cells, the nucleus and the plasma membrane.

44 Take 50 µL of cell homogenate and place it in a fresh Eppendorf On ice . This is your input sample.

LysoTag and MockTag immunoprecipitation 7m

45 Transfer the supernatant to 100 µL of the prewashed beads (step 27).

Note

The pellet is not firm and so pay extra care to not pipette out any residual insoluble material.

46 Mix by pipetting gently three times, then place on a belly-dancer orbiter for 00:05:00 at 4 °C . 5m

Note

Make sure the homogenate/bead slurry is in constant motion and the beads won't settle in any particular part of the tube.

47 Place the IPs on a tube magnet for 00:00:30 to immobilise the beads out of the supernatant. 30s

Note

Discard the supernatant or collect as flowthrough sample.

48 Resuspend in 1 mL of KBPS and immobilise the beads using the magnet for 00:00:30. Discard the supernatant. 30s

49 Repeat Step 48.

49.1 Resuspend in 1 mL of KBPS and immobilise the beads using the magnet for 00:00:30. Discard the supernatant. 30s

50 Resuspend the beads in 1 mL of KPBS and transfer to a new tube.

51 Place the tube on the magnet and after 00:00:30 discard the supernatant. 30s

52 You are now left with beads attached to lysosomes. The sample can be used for:
a. Store in -80 °C
b. Immunoblot
c. Prepared for lipidomics
d. Prepared for metabolomics
e. Prepared for proteomics