

May 07, 2024

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

 The Journal of Clinical Investigation

DOI  
[dx.doi.org/10.17504/protocols.io.261gedz2ov47/v1](https://dx.doi.org/10.17504/protocols.io.261gedz2ov47/v1)  
Daniel Saarela<sup>1,2</sup>, Dario Alessi<sup>1,2</sup>  
<sup>1</sup>Aligning Science Across Parkinson's; <sup>2</sup>MRC-PPU at The University of Dundee



Francesca Tonelli

MRC-PPU at The University of Dundee

### Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



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

External link: <https://dx.doi.org/10.1172/JCI183592>

**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>

**Manuscript citation:**

Saarela D, Lis P, Gomes S, Nirujogi RS, Dong W, Rawat E, Glendinning S, Zeneviciute K, Bagnoli E, Fasimoye R, Lin C, Nyame K, Boros FA, Zunke F, Lamoliatte F, Elshani S, Jaconelli M, Jans JJ, Huisman MA, Posern C, Westermann LM, Schulz A, Hasselt PMv, Alessi DR, Abu-Remaileh M, Sammler EM Tagless LysolP for immunoaffinity enrichment of native lysosomes from clinical samples. The Journal of Clinical Investigation 135(4). doi: [10.1172/JCI183592](https://doi.org/10.1172/JCI183592)

**License:** This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

**Protocol status:** Working

**We use this protocol and it's working**

**Created:** February 13, 2024

**Last Modified:** May 31, 2024

**Protocol Integer ID:** 95330

**Keywords:** ASAPCRN, LysoTag, lysosomes, rapid isolation of lysosome, lysosomal proteolysis, lysosome, focused investigation of lysosomal content, lysotag immunoprecipitation, lysosomal dysfunction, lysosomal content, generation of stable lysotag, stable lysotag, lysotag, protein degradation, part by protein degradation, protein, cultured cells with immunoprecipitation, turnover of protein, cultured cell, molecular homeostasis in cell, expressing cell line, proteome, magnetic bead, discovery of novel therapeutic, molecular homeostasis, cultured cells such as mouse embryonic, cell line, coupled magnetic bead, proteasome

**Funders Acknowledgements:**

**Aligning Science Across Parkinson's**

Grant ID: ASAP-000463

## Disclaimer

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to [protocols.io](https://protocols.io) is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with [protocols.io](https://protocols.io), can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.



## 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 (Clontech. Catalog# 631247).
- pGag/Pol. Lentivirus Gag/Pol plasmid. Lenti-X HTX Packaging system (Clontech. 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 Fisher Scientific Catalog #11960085**

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

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

Penicillin-Streptomycin **Gibco - Thermo Fisher Scientific 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 Scientific 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
	KH <sub>2</sub> PO <sub>4</sub>	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><sup>LINK</sup>

-  DynaMag&trade;- 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<sub>2</sub> and O<sub>2</sub> 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><sup>LINK</sup>

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

## Consumables:

-  Pierce & Warriner; Anti-HA Magnetic Beads **Thermo Fisher Catalog #88837**
-  Nunc; Cell Culture/Petri Dishes, 56.7cm<sup>2</sup>, Nunclon Delta treated, lid, vent **Thermo Fisher Catalog #172931**
-  Nunc; Cell Culture/Petri Dishes, 145 cm<sup>2</sup>, 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)

## Troubleshooting

## 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  $\mu$ L 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  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 0.45 $\mu$ m 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  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 (Step 16).





#### 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 freeze store 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  $\mu\text{L}$  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  $\mu\text{L}$  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 .
- Remove supernatant and resuspend with  100  $\mu\text{L}$  of ice cold KPBS off magnet, making sure to disperse clumps from the slurry. (1/3)



30s

26.2

- Place the tube containing the bead slurry onto a tube magnet for  00:00:30 .
- Remove supernatant and resuspend with  100  $\mu\text{L}$  of ice cold KPBS off magnet, making sure to disperse clumps from the slurry. (2/3)

30s

26.3

- Place the tube containing the bead slurry onto a tube magnet for  00:00:30 .
- Remove supernatant and resuspend with  100  $\mu\text{L}$  of ice cold KPBS off magnet, making sure to disperse clumps from the slurry. (3/3)

30s

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  $\mu$ L ), this is optimal.
- The ball size is determined by your cell type. We have found using 10  $\mu$ m gap is optimal for HEK293 and A549 cells whereas for MEF cells 12  $\mu$ m 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  $\mu$ L 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