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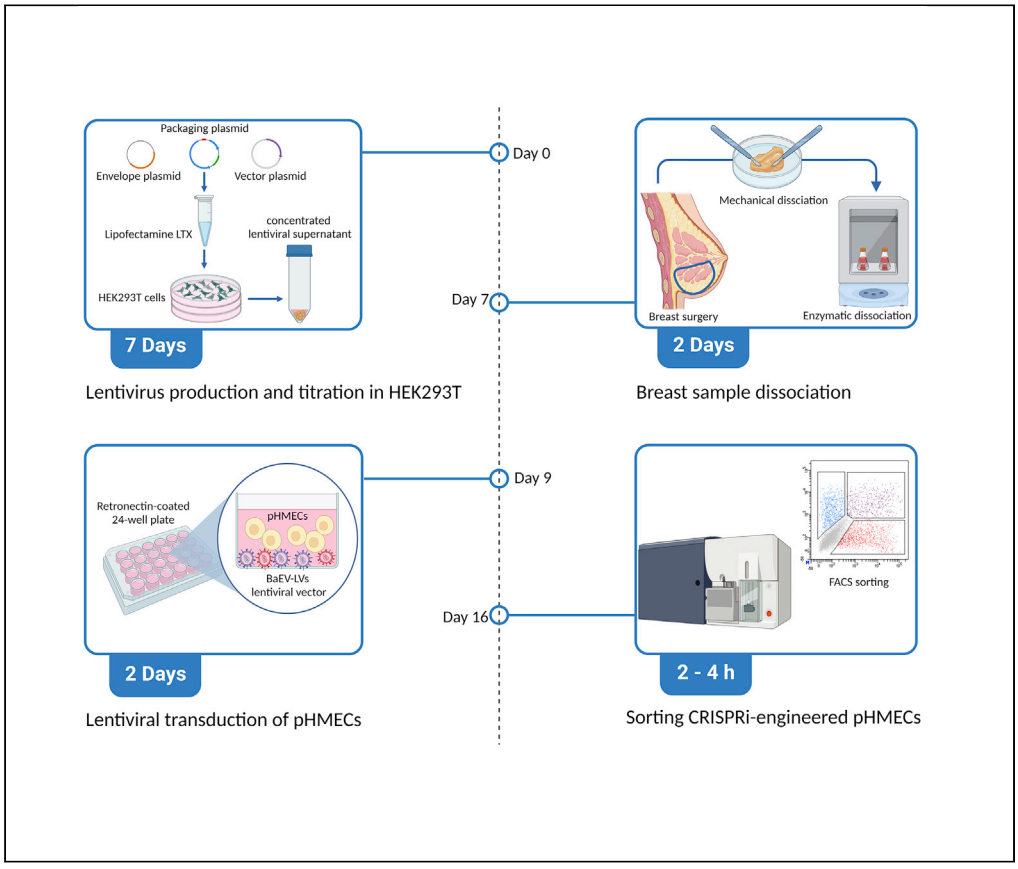
Production of CRISPRi-engineered primary human mammary epithelial cells with baboon envelope pseudotyped lentiviral vectors



Primary human mammary epithelial cells (pHMECs) are known to be remarkably difficult to engineer genetically. Here, we present a protocol for efficient transduction of pHMECs using a baboon retroviral envelope glycoprotein for pseudotyping of lentiviral vectors (BaEV-LVs). We describe the preparation of the BaEV-LVs, the isolation of pHMECs from breast samples, and the subsequent transduction of pHMECs. We also detail the use of CRISPRi technology to efficiently silence gene expression in pHMECs, which can then be used for functional assays.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol to produce baboon envelope pseudotyped lentiviral vectors

Isolation of primary human mammary epithelial cells (pHMECS) from breast samples

Engineering of pHMECs with CRISPRi technology and lentiviral transduction

Flow-cytometry-based sorting of CRISPRi-engineered pHMECs

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Production of CRISPRi-engineered primary human mammary epithelial cells with baboon envelope pseudotyped lentiviral vectors

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SUMMARY

Primary human mammary epithelial cells (pHMECs) are known to be remarkably difficult to engineer genetically. Here, we present a protocol for efficient transduction of pHMECs using a baboon retroviral envelope glycoprotein for pseudotyping of lentiviral vectors (BaEV-LVs). We describe the preparation of the BaEV-LVs, the isolation of pHMECs from breast samples, and the subsequent transduction of pHMECs. We also detail the use of CRISPRi technology to efficiently silence gene expression in pHMECs, which can then be used for functional assays.

For complete details on the use and execution of this protocol, please refer to Richart et al. (2022).[1]

BEFORE YOU BEGIN

The protocol below describes the specific steps to prepare and transduce primary human mammary epithelial cells (pHMECs) with CRISPRi lentiviral vector system. Our system is composed of two vectors, one coding for the KRAB-dCas9 fusion protein with mCherry as fluorescent reporter and the second one coding for an sgRNA targeting our candidate gene (XIST, in this study) with BFP as fluorescent reporter.[2] These vectors can be purchased from Addgene (plasmid # 60954 and plasmid # 60955). The selected sgRNA need to be introduced in the plasmid pU6-sgRNA E1Alpha-puro-T2A-BFP.

These baboon envelope pseudotyped lentiviral vectors can also be used similarly to engineer primary human T, B cells, NK cells or hematopoietic cells.[2–7]

Experimental design consideration

All experiments involving lentiviral particles must be performed under appropriate biosafety containment. The nature of the transgene must be taken into account when establishing biosafety requirements. Our work has been performed in biosafety level-2 laboratories. BSL-2 laboratories, among other things, require: (1) appropriate personal protective equipment (PPE) is worn, including

lab coats and gloves, (2) all procedures that can cause transduction from aerosols or splashes are performed within a biological safety cabinet (BSC), (3) an autoclave or an alternative method of decontamination is available for proper disposals. For more info, please refer to this link: https://www.cdc.gov/training/quicklearns/biosafety/).

Institutional permissions

Patients undergoing reduction mammoplasties have to write and sign consent to allow the use of biopsies for research practice in compliance with local regulation. Moreover, an IRB (Institutional Review Board) protocol has to be approved by the medical center performing surgery. In this study, mammoplasty samples were manipulated according to IRB protocols of the Paoli-Calmettes Institute (Marseille, France).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
Human Embryonic Kidney HEK293T cells (less than 10 passages)	ATCC	Cat#CRL-3216; RRID: CVCL_0063
Biological samples		
Mammoplasty samples from non-menauposal women (from 20 to 43 years old)	This study	N/A
Recombinant DNA		
CMVBaevTR	Els Verhoeyen Lab, C3M, Nice, France[7]	N/A
pHR-SFFV-KRAB-dCas9-P2A-mCherry	Addgene	A gift from Jonathan Weissman (Addgene plasmid # 60954 ; http://n2t.net/addgene:60954 ; RRID:Addgene_60954)
psPAX2	Addgene	A gift from Didier Trono (Addgene plasmid # 12260 ; http://n2t.net/addgene:12260 ; RRID:Addgene_12260)
pU6-sgRNA E1Alpha-puro-T2A-BFP	Addgene	A gift from Gilbert L.A. (Addgene plasmid # 60955, https://www.addgene.org/60955/)
XIST-targeting single guide RNAs:sgXIST-BFP	This study	available upon request
control-targeting single guide RNAs:sgCTRL-BFP	This study	available upon request
Chemicals, peptides, and recombinant proteins		
Fixable viability dye eFluor 780	Thermo Fisher	Cat#65-0865-14
Collagenase, Type III	Worthington	Cat#LS004183
F-12 Nut Mix (1X) + Glutamax (F12)	Gibco	Cat#31765027
DMEM, high glucose Dulbecco's Modified Eagle's Medium (DMEM)	Gibco	Cat#41965039
Formaldehyde solution	Sigma-Aldrich	Cat#252549
Lenti-X Concentrator	Takara Bio	Cat#631231
Lipofectamine LTX and PLUS reagent	Invitrogen	Cat#15338100
Opti-MEM I Reduced Serum Medium	Gibco	Cat#31985047
Fetal bovine serum qualified (FBS), heat inactivated)	Gibco	Cat#10270106 lot 42F8481K
Poly-L-lysine 0. 01% (w/v) solution	Sigma-Aldrich	Cat#P4707
Protamine sulfate salt from salmon	Sigma-Aldrich	Cat#P4020
Insulin Humalog	Lilly	VL7510 Pharmacy
Hydrocortisone	Biocodex	PC#03400933572341
HEPES	Life technologies	Cat#15630056
Antibiotic-Antimycotic	Gibco	Cat#15240062
DPBS (1X), no calcium, no magnesium	Gibco	Cat#14190-094
Dimethyl sulfoxide (DMSO) suitable for cell culture	Sigma-Aldrich	Cat#D4540
Dispase	Stemcell technologies	Cat#07913
Trypsin-EDTA (0.25%) phenol red	Gibco	Cat#25200072

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Trypsin-EDTA (0.05%) phenol red	Gibco	Cat#25300054
Retronectin	Takara	Cat#T100A
MEBM Mammary Epithelial Basal Medium	Lonza	Cat#CC-3151
B-27 Supplement, serum free	Gibco	Cat#17504044
Human EGF recombinant protein	Gibco	Cat#PHG0311
2-Mercaptoethanol 50 mM	Gibco	Cat#31350010
DNAse I solution	STEMCELL Technologies	Cat#07900
Geneticin selective antibiotic (G418 sulfate) (50 mg/mL)	Life Technologies	Cat#10131035
Other		
100 mm TC-treated cell culture dish	Falcon	Cat#353003
Minisart NML syringe filter, 0.45 µm surfactant-free cellulose acetate, 28 mm, sterile	Sartorius	Cat#16555K
150 × 15 mm not TC-treated bacteriological Petri dish	Falcon	Cat#351058
PP powder funnel	Dutscher	Cat#391495A
Sterile scalpel n°23 Swann Morton	Dutscher	Cat#132623SW
Filter unit (PES), 0.22 µm	MERCK MILLIPORE	Cat#S2GPT05RE
500 mL PP centrifuge tube, sterile	Corning	Cat#431123
White plastic box with lid	VWR	Cat#MULC25685
50 mL PP centrifuge tube, conical bottom, sterile	Falcon	Cat#352070
40 µm cell strainer, blue, sterile	Falcon	Cat#352340
KOVA Glasstic Slide 10 with Grids (Malassez Counting Chamber)	KOVA INTERNATIONAL	Cat#87144E
6-well flat bottom TC-treated plate, with lid, sterile	Falcon	Cat#353046
24-well flat bottom TC-treated plate, with lid, sterile	Falcon	Cat#353047
15 mL PP centrifuge tube, conical bottom, sterile	Falcon	Cat#352096
6-well flat bottom ultra-low attachment plate, sterile	Corning	Cat#3471
Filcon, sterile, cup-type	BD Biosciences	Cat#340625
Single-use PP syringe, 10 mL	TERUMO	Cat#8SS10ES1
5 mL round bottom PS tube with snap cap, sterile	Falcon	Cat#352054
Trypsinizing flask 2 L	BellCo	Cat#1992-02000
Stainless steel tray	Dutscher	Cat#442110
Universal scissors in stainless steel 420 - pointed tip - blade length 100 mm - total length 200	Dutscher	Cat#114538
Tweezers rounded pointed ends (145 mm)	Dutscher	Cat#076103
Aluminum foil with dispenser	VWR	291–0045
Sterile gloves	Ansell	Cat#062057B
Cut resistant gloves	Superiorglove	Cat# Dexterity® S10LXPB
Fluorescence microscope	NIKON	NIKON ECLIPSE TS2
Flow cytometer	Becton Dickinson	BD FACSAria™ III SORP
Software and algorithms		
FlowJo v. 10 Software	FlowJo (Becton Dickinson)	https://www.flowjo.com/

MATERIALS AND EQUIPMENT

Solution and medium used

All equipments and materials need to be sterilized or autoclaved. Any reagents or liquids that cannot be autoclaved need filter (0.22 µm) sterilization. We recommend all materials to be prepared freshly and stored at 4°C for no longer than two weeks. Scaling up and down according to the amount of medium needed.

Note: the batch of FBS is extremely important and might be the main source of low titers with the BaEV envelope gp; it is recommended to test several different sources of FBS and select the one that gives good titers for BaEV-LVs.

HEK Cell culture medium		
Reagent	Final concentration	Volume
DMEM	N/A	45 mL
Fetal Bovine Serum (FBS)	10%	5 mL
Total	**N/A**	**50 mL**

Note: Store complete medium at 2°C–8°C for up to 2 weeks. Before use, it is recommended to warm up the medium at 20°C–25°C.

Protamine sulfate solution		
Reagent	Final concentration	Volume
Protamine Sulfate	N/A	2 mg
Milli-Q water	N/A	1 mL
Total	**2 mg/mL**	**1 mL**

Note: Once prepared, aliquot by 100µL in 0.5 mL conical tube and store at −20°C up to one year.

Retronectin		
Reagent	Final concentration	Volume
Retronectin 1 mg/mL	N/A	0.5 mL
DPBS (1X)	N/A	23.5 mL
Total	**40 µg/mL**	**24 mL**

Note: Once prepared store at −20°C for months. Freezing and thawing can be repeated up to 4 times.

Insulin Humalog		
Reagent	Final concentration	Volume
Insulin Humalog 3.5 mg/mL	N/A	20 mL
DPBS (1X)	N/A	15 mL
Total	**2 mg/mL**	**35 mL**

Note: Mix the volume from two bottles of insulin using a syringe and an appropriate needle then transfer in a 50 mL tube. Store at 4°C up to one month.

Hydrocortison		
Reagent	Final concentration	Volume
Hydrocortison	N/A	100 mg
Distilled water	N/A	25 mL
Ethanol	N/A	25 mL
Total	**2 mg/mL**	**50 mL**

Note: Ethanol and distilled water are mixed and filtered at 0.22 μm before resuspension. Store at −20°C up to six months. Unlimited freezing and thawing.

EGF		
Reagent	Final concentration	Volume
Epidermal Growth Factor	N/A	100 μg
DPBS (1X)	N/A	10 mL
Total	**10 μg/mL**	**10 mL**

Note: Aliquot by 1 mL in 1.5 mL conical tube and store at −20°C up to six months. Freezing and thawing can be repeated up to 4 times.

Collagenase Type III dissociation medium		
Reagent	Final concentration	Amount
Collagenase type III	300 000 EU	X *g**
F-12 Medium	N/A	477.25 mL
DMEM Medium	N/A	477.25 mL
Fetal Bovine Serum (FBS)	N/A	20 mL
Insulin Humalog 2 mg/mL	10 μg/mL	5 mL
Hydrocortisone 2 mg/mL	1 μg/mL	500 μL
HEPES	N/A	10 mL
Antibiotic-Antimycotic 100X	N/A	10 mL
Total	**N/A**	**1,000 mL**

* Each batch of collagenase present is own quantity of enzyme units (EU) per *g*. It is therefore necessary to adapt the weight of collagenase to resuspend to obtain a medium containing 300 000 EU.

Note: Use a 0.22 μm filtration unit to sterilize and use fresh. Store the leftover at −20°C up to 2 months.

Freezing Medium		
Reagent	Final concentration	Volume
Fetal bovine serum (FBS)	85%	21.25 mL
Dimethyl Sulfoxide (DMSO)	15%	3.75 mL
Total	**N/A**	**25 mL**

Note: Store at 4°C up to 2 weeks.

MEBM sphere		
Reagent	Final concentration	Volume
MEBM	N/A	48.24 mL
B27 supplement	N/A	1 mL
Insulin Humalog 2 mg/mL	4 μg/mL	100 μL
Epidermal Growth Factor 10 μg/mL	2 ng/mL	100 μL
Hydrocortisone 2 mg/mL	2 μg/mL	50 μL
2-Mercaptoethanol		10 μL
Antibiotic-Antimycotic 100X	N/A	500 μL
Total	N/A	**50 mL**

Note: Store at 4°C up to 2 weeks.

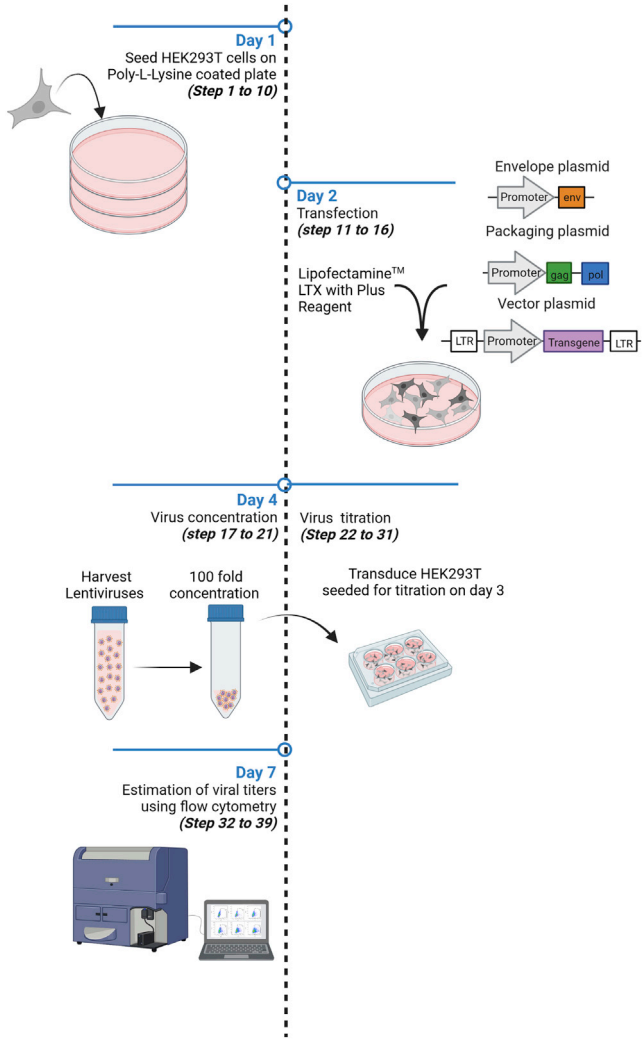

Figure 1. Workflow diagram presenting the different steps of lentivirus production

STEP-BY-STEP METHOD DETAILS

This protocol starts by the lentiviral vector production and titration (Figure 1).

Cell culture (HEK293T) preparation prior to transfection

Timing: ~7 days

Cells may take up to 7 days to expand after resuscitation and subculture. For successful lentiviral transduction, we recommend using low passages HEK293T cells after thawing (do not use cells that have been passaged more than 10 times).

Note: It is very important to keep the stock of HEK293T cells upon thawing on geneticin selection (200 µg/mL). Geneticin resistance is encoded by the SV40 large T antigen plasmid that contributes to high titers for the BaEV-LVs.

1. Rapidly thaw cryopreserved HEK293T cells by gently swirling the vial in a 37°C water bath until there is just a small bit of ice left in the vial.

2. Transfer the cell suspension to a 15 mL conical tube containing 9 mL pre-warmed HEK cell culture medium and centrifuge (200 *g*, 5 min, RT).
3. Aspirate supernatant and gently resuspend cell pellets into pre-warmed HEK cell culture medium.
   a. Count HEK293T cells using Malassez counting chamber.
   b. Seed 1.2 × 10^6 cells in a 100 mm culture dish supplemented with 10 mL HEK cell culture medium containing geneticin (200μg/mL).
   c. Incubate cell culture in a humidified incubator at 37°C containing 5% CO_2 in air.
4. Split 70–80% confluent cultures 1:4 to 1:10 for expansion.

Note: Healthy HEK293T cells show viability over 90% at all times, a doubling time of 24 h.

Lentiviral vector preparation

Timing: 7 days

In this protocol, we produce lentiviral supernatant by transiently transfecting HEK293T cells. This, using a second-generation lentiviral vector system (LentiV2), a cationic lipid Lipofectamine LTX with Plus Reagent, followed by viral particles filtration and concentration using Lenti-X Concentrator. Functional lentiviral vector titers are determined on HEK293T cells by fluorescence-activated cell sorting (FACS) analysis. This protocol has been successfully tested with different lentiviral plasmid constructs.

Using this protocol, the total yield of 100-fold concentrated Lentiviral vector achieved is usually 3–4×10^7 IFU/mL (IFU, Infectious Unit) from ten 100 mm dishes recovered 48 h post-transfection.

Lentiviral vector production

Timing: 4 days

Day 1, 18–24 h prior to transfection

5. Coat 100 mm culture dish with 2 mL of aseptic Poly-L-Lysine solution (0.01%) by gently swirling to ensure uniform coating of the plate surface.

   Note: This is optional if you use culture treated plates and not necessary if you keep the geneticin on the HEK293T cells, they will stay better adherent in that case.

6. Leave 30 min at 37°C.
7. Remove the excess solution.

   Note: Poly-L Lysine can be stored at 4°C and reused up to 5 times.

8. Thoroughly rinse the surface with sterile water at RT and aspirate (do not allow to dry at this step).

   Note: HEK293T cells detach easily from culture surface plate. To prevent producer cell detachment during wash and medium replacement steps, Poly-L-Lysine is useful to promote cell adhesion by enhancing electrostatic interaction between negatively charged ions of the cell membrane and the culture surface. After absorption to the culture surface, Poly-L-Lysine increases the number of positively charged cell binding sites.

9. Immediately plate 3–4×10^6 cells HEK293T on the Poly-L-Lysine coated 100 mm plate in 7 mL of pre-warmed HEK cell culture medium. Gently swirl the plate so that cells are uniformly seeded and incubate them at 37°C.

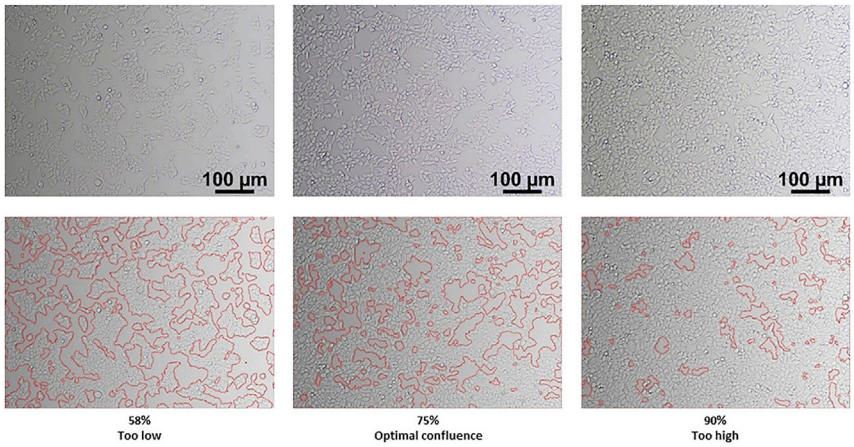

Figure 2. Estimation of cell confluence using cell confluency web tool (https://ct.catapult.org.uk/ressources/cellconfluencytool)

Note: It is necessary to find the appropriate seeding cell number to reach 70–80% confluency at the time of transfection. The optimal seeding is dependent on cell doubling time, culture medium.

⚠ CRITICAL: For transfection and titer determination, HEK293T cells should be seeded without geneticin.

Day 2, transfection

10. After 18–24 h incubation at 37°C, plates can be checked and should be ~70–80% confluency at the time of transfection (Figure 2).

⚠ CRITICAL: Cells at 90% confluency or more should not be used at this step. Indeed, this will dramatically reduce transfection efficiency.

11. Prepare the below-mentioned transfection mixtures in 15 mL conical polypropylene tubes.

Note: This mixture is suitable for one 100 mm plate. Scale up is required according to the number of plates that will be transfected.

a. Add and carefully mix the plasmids DNA in 3 mL of pre-warmed Opti-MEM I Reduced Serum Medium.

Note: Here, we recommend to use a maximal quantity of 15 µg of DNA. We used a ratio 35.5/29/35.5 to mix DNA plasmids as described in the following table:

	DNA quantity	Ratio
Packaging plasmid (psPAX2)	5.33 µg	35.5
Baboon envelop gp encoding plasmid (CMVBaevTR[a])	4.35 µg	29
Plasmid encoding for the lentiviral vector carrying the gene of interest[b]	5.33 µg	35.5

[a]CMVBaevTR, a gift from E. Verhoeyen
[b]here, sgXIST-BFP or sgCTRL-BFP or pHR-SFFV-KRAB-dCas9-P2A-mCherry

12. Add 15 μL of Lipofectamine PLUS Reagent directly to the DNA. Mix gently and incubate at RT for 15 min.
13. Add 37.5 μL of Lipofectamine LTX, mix by gentle swirling or inverting and incubate the mixture at RT for 25 min.

Note: Here, Lipofectamine LTX / total DNA ratio used is (2.5:1).

Note: The plasmids of interest express fluorescent reporter protein (mCherry or BFP). This facilitate the evaluation of transduction efficiencies and viral titers calculation by flow cytometry.

14. Transfect cells by adding 3 mL of DNA-lipid mixtures dropwise to 100 mm plate. Gently swirl the plate and incubate at 37°C.
15. After 4–6 h incubation, aspirate the media from culture dish and replace it carefully with 10 mL of pre-warmed Opti-MEM-1% HEPES. Incubate at 37°C for 48 h.

Day 3, 24 h post-transfection

16. The qualitative transfection efficiency can be validated at this step by checking expression of the fluorescent reporter protein (mCherry or BFP) using a fluorescence microscope.

Note: At this step, close to 100% of the HEK293T cells should express the fluorescent reporters indicating efficient transfection.

⚠ CRITICAL: This simple observation of the producer cells provides information about the efficacy of transfection of thus the vector production. If no fluorescence is visible at this step the transfection has failed.

Note: On Day 3 you can also execute step 22–25.

Lentiviral vector concentration

Timing: ~4 h

Day 4, 48 h post-transfection

17. Harvest viral supernatants from vector-producing cells and centrifuge (500 *g*, 10 min, RT) to remove cells and debris followed by filtering through a 0.45 μm syringe filter.

Note: If filtering, use only cellulose acetate or polyethersulfone (PES) (low protein binding) filters. Do not use nitrocellulose filters. Nitrocellulose binds surface proteins on the lentiviral vector surface and will retain the vectors on the filter.

18. Concentrate clarified lentiviral vector supernatant by adding 1 volume of Lenti-X Concentrator to 3 volumes of clarified viral supernatant (i.e., for 100 mL of clarified viral supernatant add 33 mL of Lenti-X Concentrator).
    a. mix by gentle inversion and incubate mixture at 4°C for 30 min to 16h.
    b. After incubation, centrifuge sample (1,500 *g*, 45 min, 4°C).
    c. After centrifugation an off-white pellet can be visible at the bottom of centrifugation tube.

Note: We tested incubation times ranging from 30 min to one night at 4°C without any loss of yield.

19. Gently aspirate the supernatant without disturbing the pellet.
20. Carefully resuspend the off-white pellet in 1% of the original volume using Opti-MEM-1% HEPES or medium adapted for the transduction of the target cells. For ten 100 mm dishes, 1% of the original volume (100 mL) represents 1 mL.

Note: The pellet can be somewhat sticky at first, but will go into suspension quickly.

⚠ CRITICAL: Let the sample sit for a few min with the resuspended solution. Then gently swirl or pipette up and down. Avoid fast and harsh pipetting. We recommend not to vortex.

Alternatives: Ultracentrifugation (25,000 rpm, 1h30, 4°C or 3,000 *g*, 16h) is also a common method to concentrate the viral supernatant.

21. Immediately titrate sample or store aliquots of the vector stock at −80°C until use.

⚠ CRITICAL: For long-term conservation of the BaEV-LVs resuspend the vector pellet into Opti-MEM-1% HEPES to stabilize the pH during freezing.

Lentiviral vector titration by flow cytometry

Timing: 4 days

The lentiviral vector titer is calculated by evaluating fluorescent protein expression in transduced HEK293T cells using flow-cytometry.

Day 3, 18–24 h prior to transduction

22. Count untransfected HEK293T cells using Malassez counting chamber.
23. Seed 0.2 × 10^6 HEK293T cells/well in a 6-well plate (wells numbered 1–6) and supplement with pre-warmed HEK cell culture medium to reach 2 mL final volume.
24. Gently swirl the plate so that cells are uniformly seeded.
25. Incubate 16 h at 37°C.

Note: Cells should reach 20–30% confluency at the time of transduction. Prepare six wells for transduction with serial dilutions of each vector sample. In another plate, seed in one well non-transduced control cells and another one to count the cells at the time of transduction.

Day 4, transduction

26. Aspirate the plating medium and without disturbing the cells, replace it with 2 mL of pre-warmed HEK cell culture medium.
27. Prior to transduction, prepare a stock solution of LV to be used in step 29; in a 1.5 mL tube, add 10 µL of concentrated lentiviral vector to 990 µL of HEK cell culture medium.

Note: Frozen viral supernatant should be thawed on ice. Aliquots can be freeze-thawed up to two times without altering the viral titer. Vector particles should be stored at –80°C and we advise to use them in the follow-up 3 months. If frozen in Opti-MEM-1% HEPES they are stable for over 1 year.

28. Then, in 1.5 mL tubes, perform the following vector dilutions:

Tube number	1	2	3	4	5	6
HEK cell culture medium	490 µL	475 µL	450 µL	400 µL	300 µL	0 µL
LV stock solution	10 µL	25 µL	50 µL	100 µL	200 µL	500 µL

29. Add the mixtures (tubes 1–6) dropwise to each of the seeding wells step #23 (wells 1–6).
    a. Complete with protamine sulfate to a final concentration of 10 µg/mL.
    b. Gently swirl the plate to ensure homogenous distribution.
30. Centrifuge the plate (1,000 *g*, 90 min, RT, no braking phase) to increase contact between the cells and the vector (spinoculation step).

Note: Meanwhile, count the number of non-transduced seeded cells at step #23 in the control plate. This cell count is crucial to calculate the lentiviral vector titer (transducing units/mL).

31. Place the plate in the incubator at 37°C and check the percentage of positive cells after 3 d.

Day 7, 72 h post-transduction

This part describes the sample preparation for FACS.

Note: At 3 d post-transduction, cells qualitative transduction can be checked using a fluorescence microscope.

32. 3 d post-transduction, aspirate the medium and gently wash the cells with 1 mL of DPBS (1X) avoiding to detach them.
33. Detach adherent cells by adding 0.5 mL of Trypsin-EDTA (0.25%) solution per well. Incubate for 3 min at 37°C.
34. Add 2 mL of HEK cell culture medium to each well to neutralize Trypsin.
35. Collect cells from each well in a 15 mL tube and centrifuge (500 *g*, 5 min, RT).
36. Remove the medium and wash the cells with 10 mL DPBS (1X). Centrifuge (500g, 5 min, RT).
37. Discard the supernatant and stain the cells for 10–15 min at RT with the viability dye diluted 1:1,000 in DPBS (1X) to distinguish living/dead cells.

Note: At this step it is important to use live dead cell dye to avoid misinterpretation of cell transduction.

38. Wash the cells with DPBS (1X), centrifuge (500 *g*, 5 min, RT), remove the supernatant and resuspend the cell pellet with 500 µL of DPBS (1X)-2% Formaldehyde.
39. Transfer the cell suspension into FACS tubes. Samples are ready for flow cytometry.

⚠ CRITICAL: Formaldehyde is used to fix the cells and inactivate the vector particles. Samples can be taken outside the contained laboratory for flow cytometry analysis after this step.

Note: This part describes how to calculate the lentiviral vector titer from the flow cytometry acquisition. (troubleshooting 1).

Lentiviral titer (Infectious Unit/mL) is calculated with dilutions giving 1–20% fluorescent cells with the following formula:

$$\text{Titer}\left(\frac{\text{TU}}{\text{mL}}\right) = \frac{\text{cell number} \times \frac{\%\ \text{of reporter cells}}{100}}{1/100\ \text{LV dilution (mL)}} \times \text{dilution factor}$$

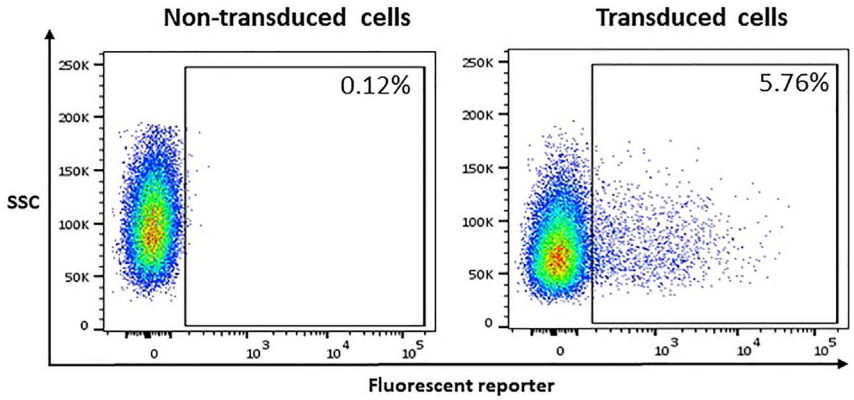

Figure 3. Gating strategy to determine the percentage of positive reporter cells by flow cytometry

For example (Figure 3), if 3×10^5 cells are transduced with 50 µL of LV stock solution (step #29 and 5.76% of positive cells are detected, the titer will be:

$$\text{Titer}\left(\frac{\text{IFU}}{\text{mL}}\right) = \frac{3 \times 10^5 \times \frac{5.76}{100}}{0.05} \times 100 = 3.45 \times 10^7 \text{ IFU / mL}$$

Note: Use only dilutions that show a linear relationship between the percentage of positive cells and the amount of lentiviral vector supernatant added to calculate the titer. The final titer should be an average of the titers obtained from transduction of at least 3 different wells of lentiviral vector supernatant.

Breast sample dissociation

Timing: 2 days

The breast sample dissociation is divided in two main steps: The mechanical and the enzymatic dissociation (Figure 4).

Note: Mammoplasty samples have to be collected by a surgeon in a sterile bag and maintained at 15°C–20°C. Dissociation has to start 3 h maximum after the surgery. Before using reduction mammoplasties samples, a pathologist has to perform sampling for medicolegal storage. This sampling has to be performed under sterile conditions. Moreover, a macroscopic analysis of mammoplasty sample by the pathologist is required to approximate the proportion of fatty tissue. This proportion is needed to adapt the dilution of dissociation medium. Before starting breast sample dissociation, make sure you have all the sterile surgical tools available for anatomic-pathological examination and tissue dissociation (Ruler, scissors, surgical tray, scalpels, and sterile aluminum foil).

Mechanical and enzymatic dissociation of breast sample

Note: During the mechanical dissociation, the experimenter needs to wear sterile gloves on top of cut-proof gloves.

40. Proceed to a mechanical dissociation of breast sample:
    a. Divide the sample in several large tissue pieces (≈100 × 50 mm).
    b. Tape dish (150 mm diameter) to the hood workbench (Figure 5).
    c. Transfer tissue pieces to taped dishes (one piece per dish) (Figure 6A).

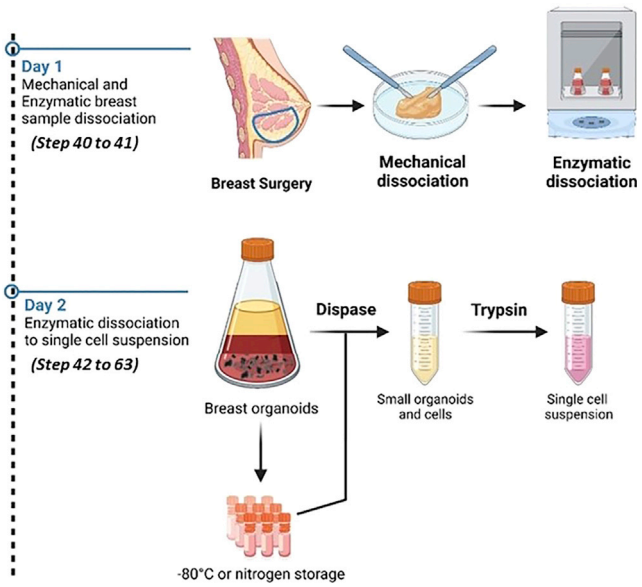

Figure 4. Workflow diagram presenting the different steps of breast sample dissociation

d. Proceed with two scalpels at a mechanical dissociation of the tissue to obtain small tissues pieces of 1–2 mm diameter (Figure 6B).
e. Change the scalpels between each dish.

Note: At this step, the time of mechanical dissociation depend on the sample size. That being said, we never exceed 3 h of dissociation to prevent cell death.

41. Transfer dissociated tissue in sterile 2 L trypsinizing flask (deflector Erlenmeyer) using a sterile funnel. Each flask should contain between 600 *g* and 800 *g* of dissociated tissue (Figure 6C).
42. Add collagenase III dissociation medium and dilute it in a solution of DMEM/F12 (1:1) according to the proportion of fatty tissue.

Proportion of fatty tissue	Volume of collagenase III	Volume of DMEM/F12
90 to 95%	375 mL	375 mL
80 to 90%	500 mL	250 mL
60 to 80%	600 mL	150 mL

43. Cover the neck of the flask with a sterile aluminum foil and seal it with parafilm (Figure 6D).
44. Place the Flask in a 2D orbital Incu-shaker (37°C, 130 rpm) for 16 h.

Note: At this step you can prepare the retronectin coated plate for 12h–16h incubation at 4°C (step #64). Alternatively, retronectin coating can be done in the incubator at 37°C for 2 h.

Collection of breast organoids and dissociation into single cell suspension

45. Following 16 h of collagenase III dissociation, allow the flask to stand on the bench for about 10 min until the suspended matter has settled in three phases (fat layer, stroma layer, organoid-enriched layer) (Figure 7A). (troubleshooting 2).

⚠ CRITICAL: Keep the flask at RT to avoid fat solidification.

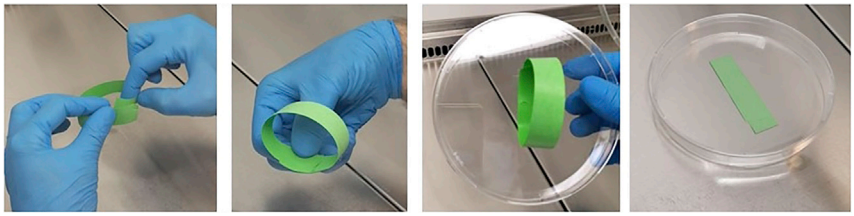

Figure 5. Preparation of dish for breast sample dissociation
Figure depicts how to tap dish on the hood workbench.

46. Allow the fat layer to drain downwards into a container by tipping the flask (Figure 7B). (troubleshooting 3).
47. Transfer the remaining liquid (stroma layer + organoids-enriched layer) in 500 mL centrifuge tube.
48. Centrifuge 500 mL tube (50 *g*, 30 s, RT).
49. Allow the stroma layer to drain downwards into a container by tipping the 500 mL tube.
50. Transfer all remaining liquid in 50 mL conical tube.
51. Centrifuge 50 mL conical tubes (80 *g*, 3 min, RT).
52. Allow the remaining liquid to drain downwards into a container being careful to retain the organoid-enriched pellet in the 50 mL conical tube.
    a. Resuspend each organoid-enriched pellet in 10 mL of DPBS (1X)-5% FBS.
    b. Pool organoid-enriched suspension in a new 50 mL conical tube (4 pellets/tube).

Note: You can check organoids size and integrity using an inverted phase microscope by transferring a fraction of the organoid-enriched suspension (250–500 μL) in a well of a 24-well plate. 25 μL of Trypan blue can be added to the suspension to improve organoid integrity analysis (Figure 8A).

53. Centrifuge 50 mL conical tubes (80 *g*, 3 min, RT).
54. Aspirate completely the supernatant without disturbing the pellet.

Note: At this step you can freeze the organoid pellets in 1.5 mL of freezing medium/pellet. We recommend using frozen organoids no later than 3 months after freezing. Cryopreserved organoids can be rapidly thawed by gently swirling the vial in a 37°C water bath until there is just a small bit of ice left in the vial. Then, transfer the cell suspension to a 15 mL conical tube containing 9 mL pre-warmed DPBS (1X)-5% FBS and centrifuge (200 *g*, 5 min, RT). Aspirate supernatant and gently resuspend cell pellets into 5 mL of pre-warmed dispase. Resume the dissociation process from step #56.

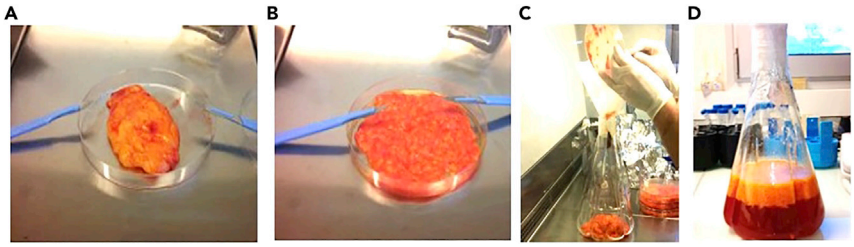

Figure 6. Key steps of breast tissue sample dissociation
(A) Representative size of breast tissue piece to mechanically dissociate per 150 mm dish.
(B) small tissue pieces obtained after mechanical dissociation.
(C) Transfer of dissociated tissue in sterile 2 L Trypsinizing Flask.
(D) Trypsinizing Flask sealed with parafilm.

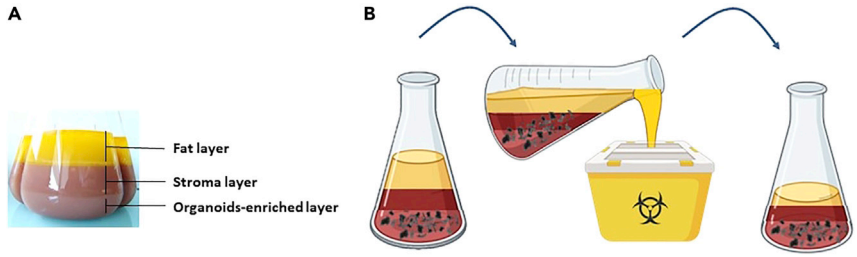

Figure 7. Layers segregation after enzymatic digestion and fat layer draining

(A) Representative layers segregation after enzymatic digestion with three distinct phases: fat layer (on the top), stroma layer (in the middle), and organoid-enriched layer (in the bottom).

(B) Schematic representation of the fat layer draining step.

55. Add 5–10 mL (depending on the size of pellets) of pre-warmed dispase to the pellet.
56. Incubate the mixture pellet/dispase at 37°C in a 2D orbital Incu-shaker for 2–3 min (130 rpm).
57. Separate the single cells from undigested organoids by pouring the mixture through a 40 μm cell strainer placed at the top of a 50 mL conical tube. Rinse the cell strainer with 10 mL of DPBS (1X).
58. Remaining organoids on the cell strainer can be transferred with disposable sterile forceps in a new 50 mL conical tube. Repeat step #55 to #57.

Note: You can check single cell suspension quality using an inverted phase microscope by transferring 10 μL of the suspension mix with 10 μL of Trypan blue in a Malassez counting chamber. If you observe only single cells you can stop the dissociation process and restart from step #64. If you still observe partially digested organoids proceed to step #59 to #63 (Figure 8B).

59. Centrifuge 50 mL conical tubes (100 *g*, 3 min, RT).
60. Aspirate completely the supernatant without disturbing the pellet.

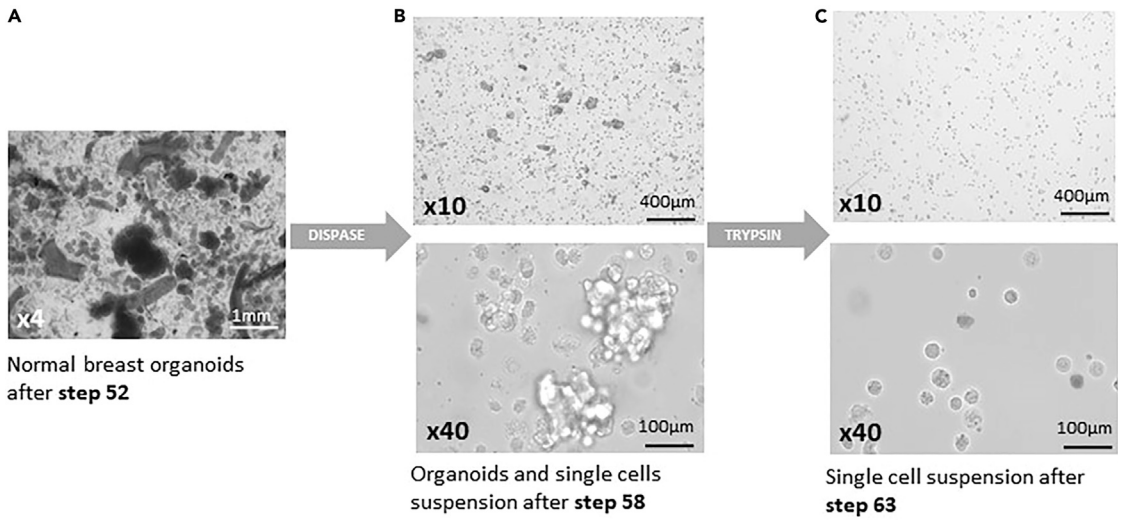

Figure 8. Representative images showing the steps of breast organoids enzymatic dissociation into single cell suspension

(A) Representative image of organoid suspension obtain at step #52.

(B) Representative image of organoids partially digested at step #58.

(C) Representative image of single cell suspension obtained at step #63.

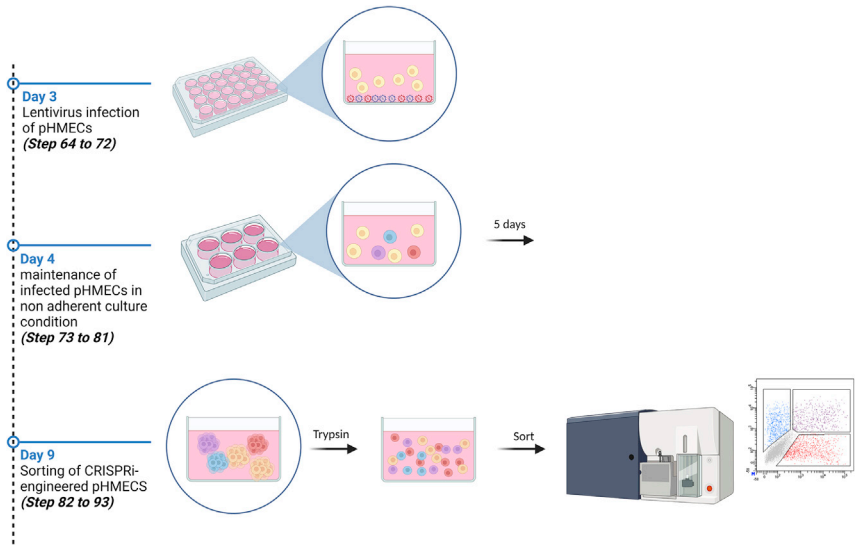

Figure 9. Workflow diagram presenting the different steps of production of CRISPRi-engineered primary human mammary epithelial cells

61. Add 2–3 mL of pre-warmed Trypsin-EDTA (0.25%) on the pellet and incubate at 37°C in a 2D orbital Incu-shaker for 2–3 min (130 rpm).
62. Add 5 mL of DPBS (1X)-5% FBS and pour the digested organoids through a cell strainer 40 μm placed at the top of a new 50 mL conical tube. (troubleshooting 4).
63. Proceed to cell counting using Malassez counting chamber (Figure 8C).

Note: We do not recommend to freeze single cell suspension of primary human mammary epithelial cells (pHMECs) for future uses. After thawing, we observed a strong proportion of damaged cells. Instead, we recommend to freeze breast organoids from step #52.

⚠ CRITICAL: Discard the container filled with fat and stroma layer in an infectious clinical waste bin sealing the lid with parafilm to avoid leakage.

Lentiviral transduction of primary human mammary epithelial cells (pHMECs)

Timing: 2 days

The production CRISPRi-engineered pHMECs is divided in three main steps (Figure 9).

Note: Pre-warm the centrifuge at 37°C for all the following step.

24-Well plate coating with retronectin

64. Add 500 μL of retronectin (40 μg/mL) in each well of 24-well plate.

Note: It is recommended to use non-tissue culture treated 24-well plates to limit cell adhesion.

65. Incubate the plate at 4°C for 12h–16h.

Note: Alternatively, retronectin coating can be done in the incubator at 37°C for 2h.

66. Collect retronectin from 24-well plate and store at −20°C (Retronectin can be reused up to three times).
67. Rinse coated wells with DPBS (1X).

Lentiviral vector transduction

68. Defrost the lentiviral vector on ice.
69. Mix both KRAB (mCherry) and sgRNA (BFP) lentiviral vectors at MOI (Multiplicity of Infection) equal to 2 in 250 µL of MEBM mammosphere medium to obtain a final volume of 250 µL. To calculate MOI use the formula below:

$$\text{MOI} = \frac{\text{lentivector Titer} \times \text{lentivector Vol}}{\text{Number of cells transduced}}$$

$$\text{Lentivirus Vol} = \frac{\text{MOI} \times \text{Number of cells transduced}}{\text{Lentivirus Titer}}$$

Note: A MOI of 2 was used in our previous study.[1] However other MOIs may be tested to increase transduction efficiency while carefully checking the cell.

70. Centrifuge 24-well plates (1,000 *g*, 3 h, 37°C, no braking phase).

Note: If you start from frozen organoids, you can defrost breast organoids and proceed to step #55 to #63.

71. Resuspend pHMECs at a cell concentration of 1×10^6 cell/mL in MEBM mammosphere medium supplemented with protamine sulfate (10 µg/mL final concentration). Add 250 µL of the cell suspension per well.
72. Centrifuge (1,000 *g*, 16h, 37°C, no braking phase).

Sorting CRISPRi-engineered primary human mammary epithelial cells

Timing: 5 days

To limit pHMECs differentiation and allow the selection of cells with an active CRISPRi system, we maintain transduced pHMEC in non-adherent culture condition.

Note: Mammary Epithelial Basal Medium must be freshly prepared.

pHMECs maintenance in non-adherent culture condition

73. Transfer non-adherent transduced cells from 24-well plate into 15 mL conical tube (2 mL/tube, i.e., a pool of 4 wells).

Note: Associate always the same wells to the corresponding 15 mL conical tube for the coming steps.

74. Rinse wells with 500 µL of DPBS (1X) and transfer the washing solution to the corresponding 15 mL conical tube containing previously harvested non-adherent infected cells.
75. To harvest adherent infected cells, add 250 µL of Trypsin-EDTA (0.05%) in each well.
76. Incubate the 24-well plate at 37°C in a cell culture incubator during 3 min.
77. Harvest cells by flushing the wells and transfer the cell suspension in the corresponding 15 mL conical tube.

78. Rinse wells with 500 μL of DPBS (1X) and transfer washing solution to the corresponding 15 mL conical tube (if some cells are still adherent repeat step #75 to #78).
79. Centrifuge 15 mL conical tubes (100 *g*, 5 min, RT) and aspirate completely the supernatant without disturbing the pellet.
80. Resuspend the pellet in 2 mL of fresh MEBM mammosphere medium and transfer each 15 mL conical tube in 1 well of Ultra-low Attachment 6-well plate.

Note: We use Ultra-low Attachment 6-well plate to avoid pHMEC's differentiation that may be induced by cell adhesion to the plate.

81. Maintain cells in culture incubator during 5 d to allow the expression of lentiviral vectors. Supplement each well every 2 d with1 mL of fresh MEBM mammosphere medium.

Mammosphere dissociation

After 5 d of culture, pHMECs generate floating colonies called mammospheres.[8] Of note, some mammospheres can aggregate together to form large 3D structures.

82. Pipet cell culture medium with mammosphere from 6-well plates and pass it through a 30 μm filcon steril cup in a 50 mL conical tube.

Note: The mammospheres will be retained on the filter and the eluate will contain only dead cells.

83. Place the filcon steril cup type at the top of a new 50 mL conical tube and turn it top side down. Plug a 10 mL syringe filled of DPBS (1X) and flush the mammosphere in the tube (Figure 10).
84. Centrifuge 50 mL conical tubes (100 *g*, 5 min, RT) and aspirate completely the supernatant without disturbing the pellet.
85. Add 2–3 mL of pre-warmed trypsin-EDTA (0.05%) on the pellet and incubate at 37°C in a 2D orbital Incu-shaker during 2–3 min.
86. Pour the digested mammosphere solution through a cell strainer (40 μm) placed at the top of a 50 mL conical tube. Rinse the cell strainer with 5 mL of DPBS (1X)-5% FBS.
87. Proceed to cell counting using Malassez counting chamber.

Preparation of transduced cells for FACS-sorting

In order to use fully active CRISPRi-engineered primary human mammary epithelial cells for future planned experiments, it is necessary to sort BFP^+/RFP^+ cells.

88. Centrifuge 50 mL conical tubes (100 *g*, 5 min, RT) and aspirate completely the supernatant without disturbing the pellet.
89. Resuspend the pellet in DPBS (1X)-5% FBS supplemented with the viability dye (dilution 1:1,000) at a final concentration of 10×10^6 cells/mL to distinguish living/dead cells.
90. Transfer cell suspension in 5 mL FACS tube (4 mL maximum volume per tube).
91. Incubate 10 min at 4°C in the dark.
92. Centrifuge 5 mL FACS tubes (100 *g*, 5 min, RT) and aspirate completely the supernatant without disturbing the pellet.
93. Resuspend the pellet in DPBS (1X)-5% FBS to obtain a final concentration of 10×10^6 cells/mL. Samples are ready for flow cytometry.

⚠ CRITICAL: At step #82 filcon cup type needs to be primed by gently tapping it on the top of 50 mL conical tube. Moreover, sometimes if there is a large amount of mammospheres the filcon sterile cup type can be clogged. Do not hesitate to use additional sterile cup type to finalize mammosphere harvesting.

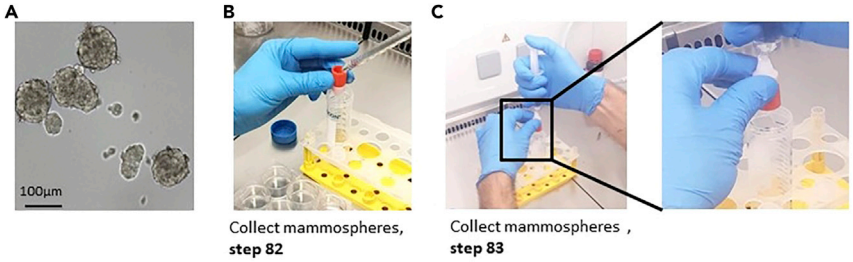

Figure 10. Key steps of mammosphere collection

(A) Representative image of mammospheres obtained after 5 d of culture.

(B) Filtering of mammosphere-containing medium through a filcon steril cup type 30 µm.

(C) Flushing of mammospheres retain on the filcon steril cup type 30 µm.

EXPECTED OUTCOMES

Typically, we obtained a lentiviral vector titer ranging from 1×10^7 to 1×10^8 IFU/mL and we were able to infect 12.5×10^6 pHMECs per lentiviral production. After 5 d of pHMECs culture in suspension the proportion of live cells resulting from mammosphere represent 4×10^5 cells. The efficiency of transduction was ranging from 12.7% to 20.4% of live cells for a transduction with a single vector (either mCherry + or BFP+) and from 5.2% to 6.2% for the insertion of both construct (mCherry+/BFP+) (Figure 11). Silencing of XIST was evaluated in sorted mCherry+/BFP + pHMECs by qRT-PCR. We were able to silence 90% of XIST expression in the CRISPRi-engineered pHMECs with sgXIST compared to pHMECs engineered to express a scrambled sgRNA.[1] Of note, the use of vector expressing two sgRNAs targeting two different genes can be considered but a decrease in the efficiency of gene expression silencing is expected.[2] This CRISPRi-engineered pHMECs were used in different functional assays *in vitro* (generation of 3D organoids) and *in vivo* (xenotransplantation in immunocompromised mice). XIST silencing was maintained during all these experiments attesting the stability of our CRISPRi system.

LIMITATIONS

Although we greatly improved the transduction efficiency of pHMECS, it remains limited and we had to repeatedly produce new lentiviral vectors to perform functional assays. Key steps of this protocol should be considered for optimization.

First, we are currently working on upgrading lentiviral production to increase titer. We are testing ultracentrifugation to concentrate the vectors or using different transfection reagents to transfect initially HEK293T cells. Indeed, since our paper was published, we successfully adapted an alternative protocol using Polyethylenimine (PEIpro® - Polyplus) as transfection agent and increase the total yield of concentrated lentiviral vectorup to 1×10^8 IFU/mL (IFU, Infectious Unit) from ten 100 mm dishes recovered 48 h post-transfection. Moreover, in this study we did not use lentiviral packaging cell lines that are commercially available (i.e., Lenti-X™ 293T Cell Line; Takara). Hence, we are also working on trying to increase the cell numbers that can be transduced without changing the quantity of vector used. To this purpose we are changing volumes, plates and media that may influence the vector production. Moreover, we used in this study retronectin and protamin to facilitate lentiviral vector entry, but other reagents such as vectofusin or polybrene are frequently used with hematopoietic cells and may improve transduction as shown by us and others.[9–11] Others also recommended multiple transduction steps or collecting the virus supernatant several times when producing it, although this is not recommended for primary cells since the titer in a second harvest goes down.[12] Finally, we may consider using a lentiviral vector co-expressing the KRAB-dCas9 fusion protein and the sgRNA. As previously observed, we divided by two the proportion of cells expressing two separate cassettes compared to the cells transduced by only one (Figure 11). The use of an "all-in-one" lentiviral vector will substantially improve our transduction rate.

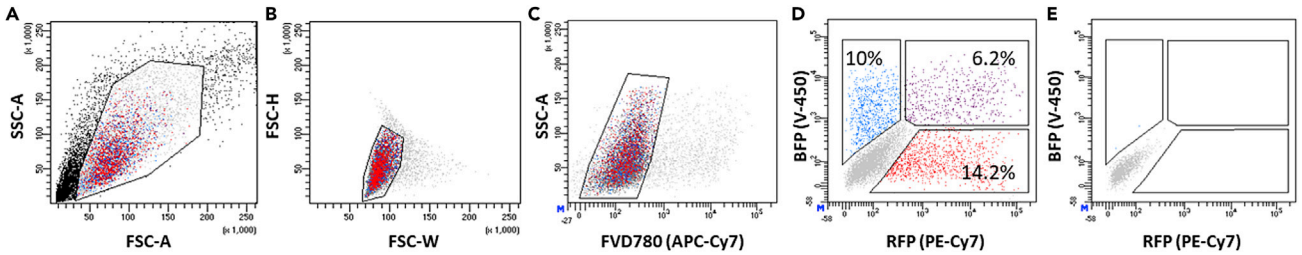

Figure 11. Characterization of CRISPRi-engineered pHMECS

Schematic representation of the gating strategy for flow cytometry used to sort pHMECs mCherry$^+$/BFP$^+$. First pHMECs are separated from debris by Forward Scatter Area (FSC-A) / Side Scatter Area (SSC-A) (A), and doublets and aggregates are excluded on FSC-Width (FSC-W) and FSC-Heigh (FSC-H) dot plots (B). Living cells are selected by gating the negative fraction for FVD eFluor780 (C). Then, the population of mCherry$^+$/BFP$^+$is gated (D) using an untransduced control condition as baseline delimiting negative and positive cells (E).

We describe here an efficient method to transduce pHMECs that were historically extremely difficult to engineer genetically. Future method improvement may refine the way pHMECs are transduced. We believe this method to be a first foundation stone for future genetic engineering of pHMECs and other cell types difficult to transduce.

TROUBLESHOOTING

Problem 1

Low lentiviral vector yield (see step 22–39).

Potential solution

Use low-passage cells and allow cells to recover completely from frozen stock (at least two passages) before transfection (steps #1–4). Addition of geneticin is crucial to keep the HEK293T cells fit for lentiviral vector production. For adherent cells, ensure that cells are at 70–80% confluency at the time of transfection. Use high-quality plasmid preparation, free of protein and RNA (OD260/280 > 1.8). Optimize the ratio between the different plasmids used and the ratio of Lipofectamine LTX to the total amount of DNA.

Problem 2

Sometimes, following 16h of collagenase dissociation, the settling time is not sufficient to observe three distinct layers (see step 45). You can only observe a homogenous brown mixture. It corresponds to an over-digested breast tissue. In that case, we strongly recommend to trash the sample that will mainly contain damaged cells.

Potential solution

This over-digestion may be due to a wrong estimate of the proportion of fatty tissue. We suggest to reduce the volume of collagenase III for similar breast tissue samples.

Problem 3

Sometimes, following 16h of collagenase dissociation, the flask still contains undigested pieces of tissue (>3 mm diameter) sunk to the bottom (see step 46). These pieces of tissue may correspond to fibrosis and will not be digested by our enzymatic digestion protocol.

Potential solution

We suggest to fish out these pieces using disposable sterile forceps during step #45.

Problem 4

During tissue dissociation, parts of the cells are lysed resulting in a release of DNA that generates floating DNA monomolecular structure (white cloud). It is sometimes the case after dispase and/or

trypsin digestion (see step 62). The DNA monomolecular structure may cause clumping of cells and limit cell pellet adhesion to the bottom of conical tube.

Potential solution

Resuspend cells in 0.1 mg/mL of DNase I Solution for 15 min at RT. Addition of DNase I leads to a degradation of this extracellular DNA, thereby avoiding the loss of cells from undesired clumping.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Christophe Ginestier (christophe.ginestier@inserm.fr).

Materials availability

No newly generated plasmids, mouse lines, or other unique materials are associated with this protocol. The BaEV envelope gp encoding plasmid is patented and available from Els Verhoeyen (els.verhoeyen@ens-lyon.fr) upon request and signature of an MTA for research purposes).

Data and code availability

No dataset were generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.P., J.W.; methodology, S.P., J.W.; figures, S.P., J.W.; writing – review & editing, S.P., J.W., E.C.-J., E.V., G.G., C.G.; resources, G.G., C.G.; funding acquisition, G.G., C.G.; supervision, G.G., C.G.

DECLARATION OF INTERESTS

E.V. is the inventor of the patent on pseudotyping of retroviral particles with BaEV envelope glycoproteins (patent WO 07290918.7).

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