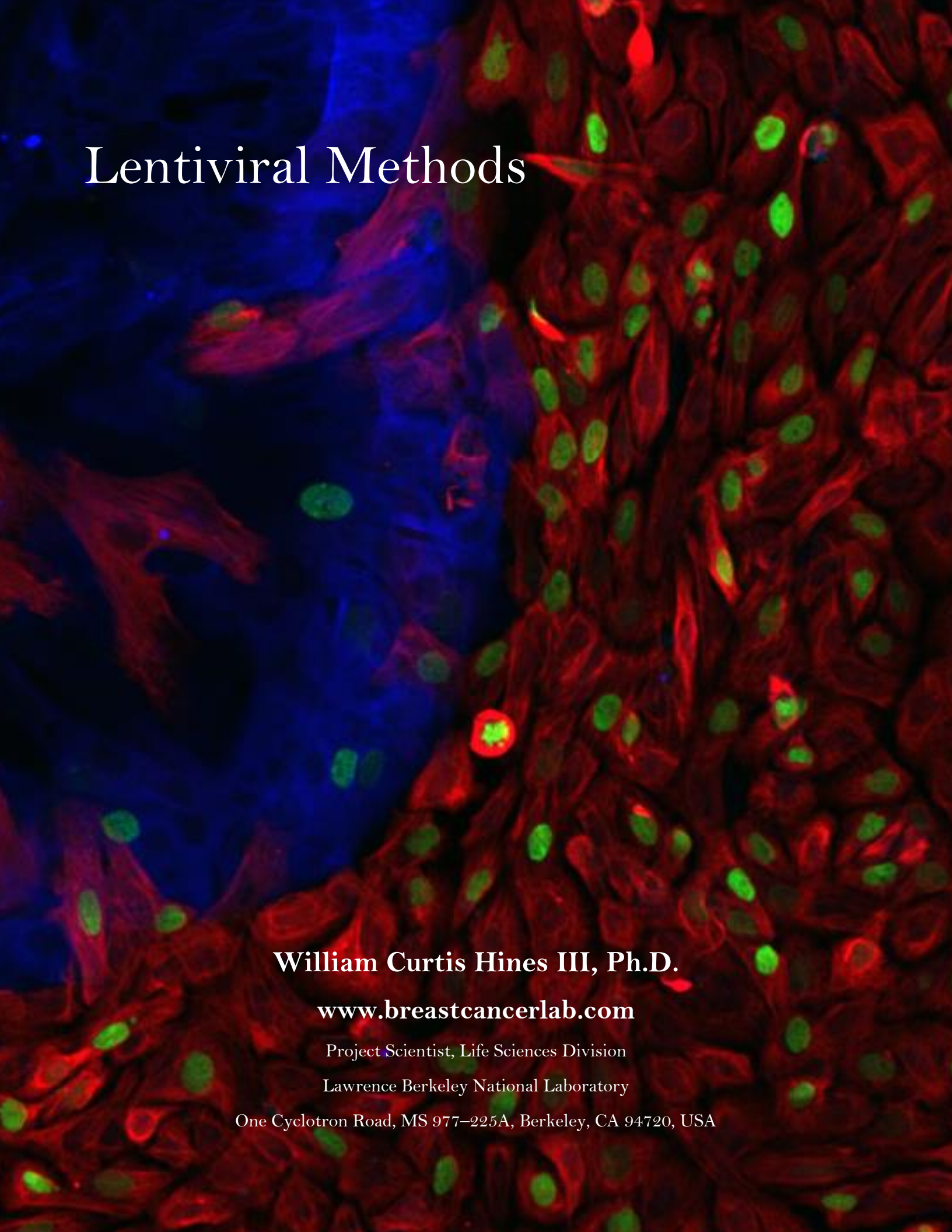
Lentiviral Methods



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Biosafety

Lentivirus Packaging Protocol

Concentrating Lentivirus Particles

Titrating Lentivirus

References:

1. Hines, W.C., Yaswen, P. & Bissell, M.J. Modelling breast cancer requires identification and correction of a critical cell lineage-dependent transduction bias. Nature communications 6, 6927 (2015).
2. TRONO website: http://tronolab.epfl.ch
3. Sena-Esteves M, Tebbets JC, Steffens S, Crombleholme T, Flake AW: Optimized large-scale production of high titer lentivirus vector pseudotypes. J Virol Methods 2004, 122(2):131-139.
4. Sutton, R. E. Production of Lentiviral Vector Supernatants and Transduction of Cellular Targets" in Methods in molecular medicine.
5. Tiscornia, G., O. Singer, and I.M. Verma. 2006. Production and purification of lentiviral vectors. Nat Protoc. 1:241-5.
6. Current Protocols in Cell Biology; 20.3 Calcium Phosphate Transfection
7. Addgene.org

Biosafety: Applicable Legislation, Standards & Guidelines

Note: Most Lentivirus work is classified as BIOSAFETY LEVEL 2

Before initiating any experiments with lentiviruses, make sure you are approved to do so and that you are following National, State and Institutional guidelines.

For more information:

- National Institutes of Health, Office of Biotechnology Activities; Recombinant DNA Advisory Committee: Biosafety Considerations for Research with Lentiviral Vectors (March 2006):

Risks of lentivirus vectors:

- The major risks to be considered for research with HIV-1 based lentivirus vectors are:
  - potential for generation of replication-competent lentivirus (RCL), and
  - potential for oncogenesis.

These risks can be mitigated by the nature of the vector system (and its safety features) or exacerbated by the nature of the transgene insert encoded by the vector.

General criteria for risk assessment of lentivirus vectors:

- Decisions about containment should take into account a range of parameters/considerations including:
  - the nature of the vector system and the potential for regeneration of replication competent virus from the vector components,
  - the nature of the transgene insert (e.g., known oncogenes or genes with high oncogenic potential may merit special care)
  - the vector titer and the total amount of vector,
  - the inherent biological containment of the animal host, if relevant,
  - negative RCL testing

General containment considerations:

- Either BL2 containment or enhanced BL2 containment is often appropriate in the laboratory setting for research involving the use of advanced lentivirus vector systems that have multiple safety features and that segregate vector and packaging functions onto four or more plasmids. Enhanced BL2 containment may include in addition to attention to sharps (and use of safety needles where feasible), the use of personal protective equipment intended to reduce the potential for mucosal exposure to the vector. In most such research, these levels of containment are also expected to be appropriate even when producing large volumes of HIV-1 vectors (>10 L).

- The appropriate containment level for specific lentivirus vector research is, of course, determined following a complete risk assessment and local IBC review. The following sections discuss some considerations which should form an important part of the biosafety assessment for research involving lentivirus vectors.

- A comprehensive risk assessment and determination of containment for research with lentiviral vectors should consider the nature of the vector system, transgene insert, and type of manipulations involved. For many experiments, either BL-2 or enhanced BL-2 will be appropriate.

Lentivirus Packaging Protocol

Materials:

- HEK293FT cells (maintained in DMEM 10% FBS + Penstrep)
- Packaging Plasmids
  (pLP1,pLP2,pLP-VSVG, for 3rd Gen system, see below)
- 15cm culture dishes
- HEK medium: DMEM+10%FBS (3-4 bottles)
  - DMEM High Glucose (e.g.Invitrogen 10564-029)
- Calcium Phosphate Transfection Buffers (see below)
- 0.45 µm Nalgene filtration units
- Ultracentrifuge tubes (Beckman 358126)
- Ultracentrifuge tube adapters
- Poly-L Lysine 0.001% in PBS (optional)

A note about lentiviruses and packaging constructs:

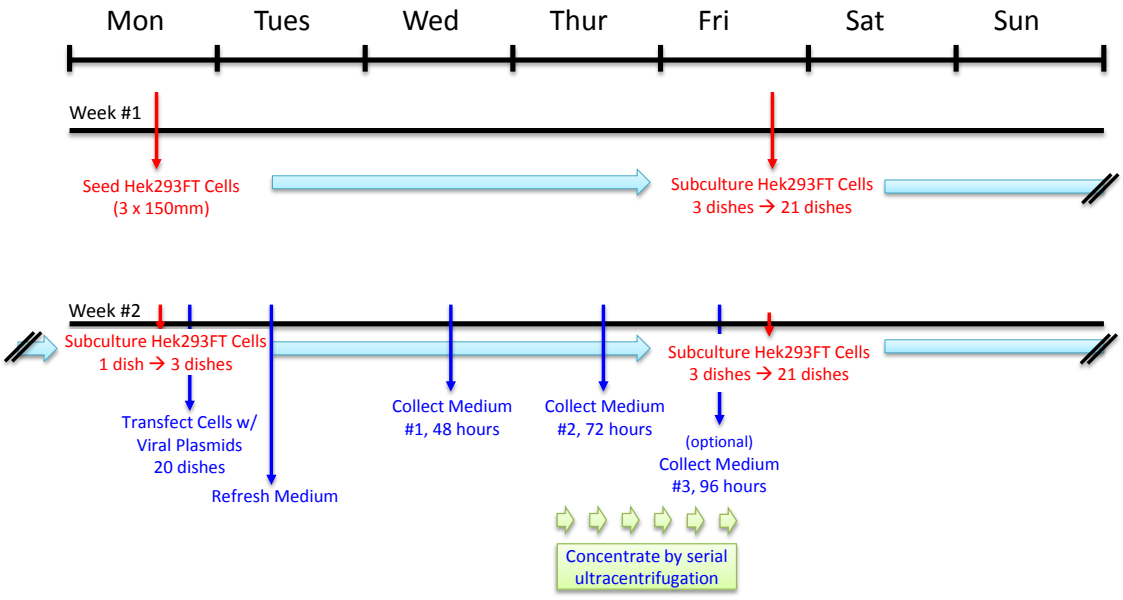
Lentivectors carrying a chimeric 5'LTR (e.g., CCL, RRL,..etc) can be packed in either a 2nd or 3rd generation packaging system; however, those that have a native HIV 5'LTR (e.g., LVTH, pWPI, pWPTS, pWPXL) must be packaged with a 2nd generation system (these require TAT expression in producer cells).

	2nd Gen	3rd Gen
Gag/Pol/tat/REV	psPAX2 or pCMV-dR8.2 dvpr	
Gag/Pol		pLP1 or pMDLg/pRRE
REV		pLP2 or pRSV-Rev
VSVG	pMD2.G or pCMV-VSVG	pLP-VSVG or pMD2.G
Lenti transfer vector	LVTH, pWPI, pWPTS	pLENTI, CCL, RRL, pLKO.1

Before you get started:

- Double-check and ensure you have all the required reagents! 3 x 150mm plates of Hek293FT cells, buffers made, plasmid preps,etc.
- PLASMIDS: Make sure you have enough Plasmid DNA (Lentiviral transfer and Packaging constructs) for the following week's transfection. (If not, inoculate broth and do plasmid preps!)
- Expanding HEK293FT cells takes 1 week and the process of transfection, collection, and concentration takes 1 week:

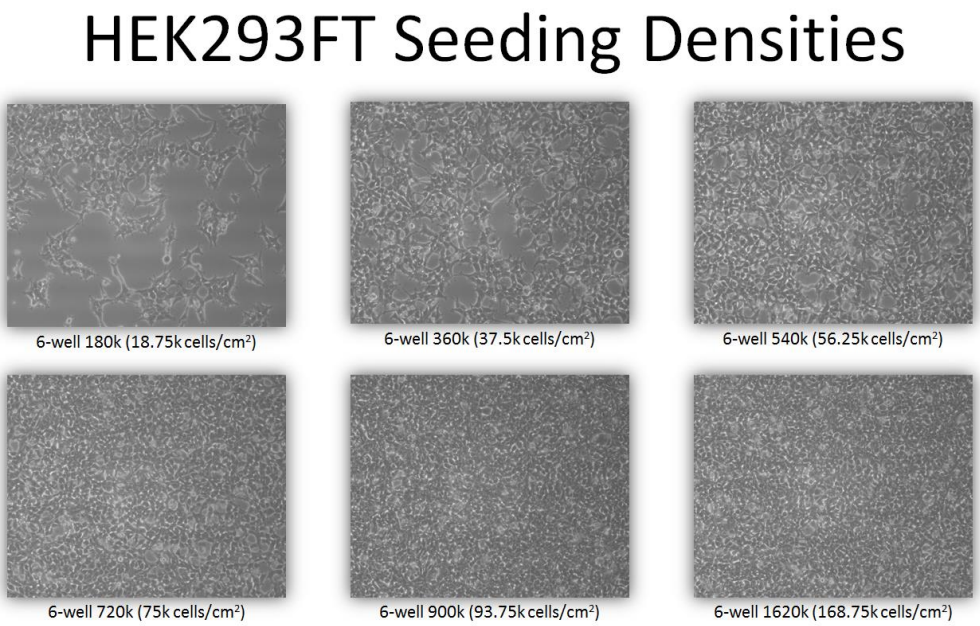
Overview of the procedure:



Lentivirus Packaging Protocol

Day One (Friday): Seed Cells

- Trypsinize 3 x 150mm 90% confluent dishes of Hek293FT cells and:
    - Seed 21 x 150mm dishes with these cells (cells will be ready to transfect on Monday, 72-96 hours after the split)
    - Or…count the 293T cells, and seed 2.5e7 cells per 15 cm tissue dish to obtain 90–95% confluent culture the following day.



- Reserve Ultracentrifuge for the upcoming Thursday. Depending on how often it is being used, you will have to decide to either put up sign or send an e-mail to the group's list-serv (a sign is usually sufficient).

Day Four (Monday, Late afternoon): Calcium Phosphate Transfection

- The HEK cells should be around 70% confluence today. Wait until the end of the day to transfect them to make it convenient to refresh the medium, which should occur 12-16 hours post transfection (the following morning).
- 2 hours prior to transfection **(~3:00 PM)**
    - Preheat the medium to room temp: remove from fridge and place it on the counter. Let it sit for 30-60 min.
    - **Split** one 150mm dish into 3x 150mm dishes to continue the virus preparation cycle. (If we are to package virus next week, we will need 3 x 150mm 90%-confluent dishes of HEK293FT this upcoming Friday).
    - **Feed** 20x150mm dishes of cells roughly 1-2 hours before transfection with 15mL of fresh pre-warmed medium.
- Time of transfection **(~5:00 PM)**

    -Refer to Calcium Phosphate Transfections Method (in the appendix)

    - Thaw 2XHBS and $CaCl_2$ (this is in the -80°C Freezer)

Lentivirus Packaging Protocol

- Prepare Plasmid mixes (Excel Worksheet):

								1
	bp	Conc. ng/µL	per 15cm dish (ug)					Volumes (ul)
	number of 150mm dishes:							20
Plenti #1 Plasmid		300	18					1200.0
pLP1 (gag/pol)	8889	450	18					800.0
pLP2 (Rev)	4180	500	8.5					340.0
Envelope GP (pLP/**VSVG**)	5821	600	13					433.3
Volume of DNA =								2773.3
TUBE A (Mix Water, Calcium Chloride, DNA) Use a 50mL conical tube				**1 x 150mm (ul)**				
		Add:	**2.5 M $CaCl_2$ (0.25M final)**	79.5		**$CaCl_2$**	1,590.00	
			water			**Water**	11,536.7	
			TOTAL(q.v.)=	795			15,900.0	
TUBE B USE a 50mL conical Tube		**Bubble "A" into this volume:**	Hepes buff. Saline (HBS)	795			**15900.0**	
				Each dish will get 1590ul of precipitate				

- **SLOWLY** add solution "A, DNA+CaCl2" dropwise to tube "B, Hepes buffer" while bubbling air through solution "B" with another pipette. Continue until solution "A" is depleted. This is a slow process, and should take several minutes.
- Incubate at room temp for **15-30 min**. A fine precipitate should form.
- After the incubation, add the precipitate dropwise to the medium/cells in the 150mm dishes (1590 µL per 150mm dish).
- Gently rock the dishes back-and-forth. Place in incubator

Day Five (Tuesday, early morning): Refresh Medium

CAUTION: VIRUS IS BEING MADE AND IS ALREADY PRESENT AT THIS STAGE: Remember to use Biosafety level 2 procedures.

- At 12-16 hours post-transfection, remove medium (containing precipitate) and replenish with **15ml/dish** of fresh pre-heated (room temp) medium (DMEM/10%FBS).

Day Six (Wednesday morning): Virus Collection #1

- **Collect virus-containing medium:** Collect medium from the plates and place into 6 x 50ml tubes. Add fresh medium to the cells (15ml/dish).
- Centrifuge the virus-containing medium to pellet cell debris (1000-2000g for 5 minutes). When it is done, place the supernatant into a disposable 500ml sterile Nalgene Receiver bottle (bottles that have never been in contact with bacteria/endotoxins). Store at 4°C until you are ready to concentrate the virus (day 7).

Day Seven (Thursday morning): Virus Collection #2

- **Collect virus-containing medium:** (repeat collection as on day 6)

Lentivirus Packaging Protocol

- **Filter supernatant through a 0.45um Nalgene filtration unit.** Centrifuging the cell debris will prevent clogging of the filter. (Note: using a 0.2μm filter is small enough to damage viral particles and will thus decrease effective yield)
- **Begin virus concentration by ultracentrifugation (or other method, see instructions below)**

Day Eight (Friday morning): Virus Collection #3

- **Collect virus-containing medium:** (repeat collection as on day 6)
- **Continue virus concentration by ultracentrifugation**

- **Disinfect and discard dishes:**
    - BIOHAZARD-remember these dishes still have virus in them!
    - To discard, add bleach (10%) to the dishes,let sit for 1` min, then suck up bleach with vacuum. Discard dishes in biohazardous waste. Change out the biohazard bags.

Buffers

1. CaCl2, 2.5 M
    - 183.7 g CaCl2·2H2O (Sigma; tissue culture grade)
    - H2O to 500 ml
    - Filter sterilize through a 0.45-µm nitrocellulose filter (Nalgene)
    - Store at −20°C in 10-ml aliquots
    - This solution can be frozen and thawed repeatedly.

2. HEPES-buffered saline (HeBS) solution, 2×
    - 16.4 g NaCl (0.28 M final)
    - 11.9 g HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; 0.05 M final)
    - 0.21 g Na2HPO4 (1.5 mM final)
    - 800 ml H2O
    - Titrate to pH **7.05** with 5 N NaOH
        - 12/12/2011: Note that the new HBS with a pH of 7.05 did not perform as well as the old buffer which had a ph of 7.19. We prepared another new prep of buffer and titrated it to a pH of 7.19 to match the old buffer. The transfection worked well this time. So, use a pH of 7.19 (preferably –due to calibration of the meters, it would be a good idea to save some old buffer you know works well and match the pH of that buffer to the new.
    - Add H2O to 1 liter
    - Filter sterilize through a 0.45-µm nitrocellulose filter
    - Test for transfection efficiency
    - Store at −20°C in 50-ml aliquots
        - An exact pH is extremely important for efficient transfection. The optimal pH range is 7.05 to 7.12. There can be wide variability in the efficiency of transfection obtained between batches of 2× HeBS. Efficiency should be checked with each new batch. The 2× HeBS solution can be rapidly tested by mixing 0.5 ml of 2× HeBS with 0.5 ml of 250 mM CaCl2 and vortexing. A fine precipitate should develop that is readily visible in the microscope. Transfection efficiency must still be confirmed, but if the solution does not form a precipitate in this test, there is something wrong. Another test for the precipitate is to shine a laser pointer through a 15 ml tube of ~5ml of the precipitate.

3. 5N NaOH
    a. Dissolve 6grams of NaOH pellets in 24ml water. After they dissolve, adjust volume to 30ml.

Confirm ability of buffers to transfect HEK293FT cells

To check the ability of new buffers to transfect HEK293 cells:

- Seed 75,000 HEK293FT cells into a 24 well dish, with 300ul culture volume
    - Tube A: 0.5 ug of DNA (1.4ul)+2.04ul Cacl2 + 16.9 water = 20.4 ul
    - Tube B: 20.4 ul of 2x HBS
- Vortex together and Let sit for 30 min.
- Add 40.8ul to the cells and observe transfection the following morning (GFP and presence of syncytium)

Virus Concentration

- This procedure concentrates live virus, so as always, perform work in a class II biosafety hood.
- Remember that the concentrated virus will ultimately be used to infect cell cultures, so use sterile tubes and conditions.

Concentration by Ultracentrifugation

- Centrifugation tubes: we use conical tubes (as opposed to round bottom tubes) and swinging bucket rotors (as opposed to fixed angle rotors). These are recommended because they make the lentiviral pellet easy to see. We have a **Beckman SW28 rotor,** which requires Beckman 358126 tubes. **IMPORTANT**: conical tube adapters are also required.
- If not already performed, clear and filter the virus-containing medium through a 0.45µm filtration unit. To prevent clogging, first clear the medium of cellular debris by centrifuging it 2000g for 5 minutes, and pour supernatant into a bottle or into a 0.45um filtration unit.
    - Sterilize the ultracentrifuge tubes (Beckman 358126) by rinsing them with 70% ETOH. Rinse twice with PBS to remove ETOH. (DO NOT AUTOCLAVE!- it weakens the tubes).
- Fill the tubes with 26 ml of supernatant.
- To the bottom of each Beckman ultra centrifuge tube, add 2 mL of 20% Sucrose Solution using a serological pipette.
    - 20% Sucrose Solution (per 500 mL):
        - - 100 g sucrose
        - - Bring the volume to 500 mL using PBS without Ca2+ or Mg2+ D-PBS
        - - Filter with 0.22 um filter unit
- **BIOHAZARD: Cap all tubes before removing from the Biohazard Safety Cabinet/Hood.**

Weigh the tubes, and balance opposite tubes to nearest milligram (0.1 gram). **THIS IS VERY IMPORTANT, AND IF YOU DON'T DO THIS,THE ULTRACENTRIFUGE WILL DETECT AND GIVE AN "TUBE IMBALANCE" ERROR.** IF THIS HAPPENS, YOU WILL HAVE TO REMOVE VACUUM, WEIGH THE TUBES AND REPEAT –this all takes time. Weigh your tubes!

- **Apply the vacuum, set brake to stop @ 800 r.p.m., and set the centrifuge to spin @ 26,000 r.p.m.** for **at least 90 (90-120) minutes at 4 °C**.

The total volume of supernatant from a 20 × 15 cm dish preparation is 600 ml; therefore, processing the full volume will **require 3.5 (4) consecutive spins**. On the last spin, we will only need 2 tubes, so consolidate the 6 pellets to two tubes (using supernatant, PBS or HBSS). Also**, at all times make sure the tubes are full (have 28 ml - or the tubes will crush themselves during the spin** (add PBS, if needed)).

- After the last spin, pour off the supernatant into a waste container. Aspirate excess liquid on the side of the tube. Invert the tubes on paper towels. Siphon off remaining liquid without disturbing the pellet. The pellet should be barely visible as a small translucent spot (looks like a contact lens) –it is sometimes yellowish and looks greasy.
- Resuspend the viral pellets in 100 µl of 1× HBSS (or PBS). **Avoid frothing**. Rinse the tubes with another 100 µl of 1× HBSS. Repeat as necessary. Pool both volumes to obtain 400 µl final volume. The virus will have a milky appearance. Make sure the pellet does not stick/remain on your pipette tip!
- Transfer to a screw-cap microfuge tube, and vortex at low speed (room temp) for 15–30 min. The resuspended viral preparation will have a clear to slightly milky appearance.
- Clear the suspension by spinning for 2 minutes on a tabletop microcentrifuge (5,000 rpm). Transfer the supernatant to a fresh screw-cap microfuge tube.
- Make 10-20 µl aliquots of the supernatant. This viral supernatant is *in vitro* grade quality. STORE virus @ -80°C.
- Print-out labels, add place them on the tubes, record how many tubes were made and where they are located in our virus inventory sheet.
- Titrate the virus after it has been frozen, as this is what will be used in the assays

Treating your virus with Neuraminidase

We have found treating concentrated lentivirus with neuraminidase improves the transduction of primary luminal breast cells while also increasing the effective titer of the virus (Hines et.al., 2015). This is a quick and easy procedure and can be performed either before or after the lentivirus is frozen and archived.

Materials Needed: Neuraminidase from Vibrio cholerae, type III (Sigma, N7885-2UN)
Note that there is lot-lot variability in the specific activity of the enzyme preparations. Nevertheless, the amount required to treat the virus (200mU/ml) typically requires ~ 1:50 dilution.

We usually concentrate 600-900ml of virus and resuspend in 200ul Hanks Balanced Salt Solution (HBSS). The amount of HBSS required to dissolve the entire virus pellet will depend on the yield of the virus preparation however. We then spin the virus (now in a sterile screw-cap microcentrifuge tube) at 8,000g x 3min. After the spin, we transfer the virus-containing supernatant to a fresh tube–leaving debris behind. Measure the volume or q.v. 200ul with HBSS. Add ~1:50 volumes (4ul) of Neuraminidase (final concentration of 200mU/ml) and incubate at 37°C for 45 mintues. After the incubation, make 10-20ul aliquots and place in the -80°C freezer for storage.

Hines, W.C., Yaswen, P. & Bissell, M.J. Modelling breast cancer requires identification and correction of a critical cell lineage-dependent transduction bias. Nature communications 6, 6927 (2015).

Titrating your virus

If your virus expresses a fluorescent protein, then you can easily assess viral activity by treating a sensitive cell line with your virus and monitor for fluorescence, ideally by flow cytometry.

To determine if your virus preparation was successful and gauge the titer of the virus, incubate the virus with a sensitive cell line. I prefer MDA-MB-468 cells, because they are the most lentiviral-sensitive cell line that I have tested to date.

It is absolutely necessary to also test your virus on your cells of interest (and under identical conditions you will ultimately use in your experiments).

Cells have a wide-range of viral sensitivities, so the amount of virus needed to achieve a specific level of transduction for a specific cell type will have to be empirically determined. For more info on effects of FBS and polybrene, and the relationship between transduction efficiency and viral integrations, refer to: [1] **Modelling breast cancer requires identification and correction of a critical cell lineage-dependent transduction bias. Nat Commun, 6, 6927.**

- **DAY1:**
  - **Seed 50,000 MDA-MB-468 cells** into each well of a 24-well dish 24 hours prior to infection (720,000 cells in 12 mL). This number of cells will permit 3 days of growth.
- **DAY2:**
  - Confirm the cells have attached, then refresh the medium (300µl per well)
  - **Make 2-fold serial dilutions of the lentiviral preparation in 300µL total volume**
    - Add 300µl to the first well, so the total volume is 600µl in this well
    - Add 5-10 µL of concentrated virus to the first well (600µL) and thoroughly mix (pipette 300µl up and down 10x)
    - Remove 300µl from first well and add to second well and thoroughly mix.
    - Repeat to the last well. (remove and discard 300µL from the last well)

600ul (+10µL virus)	300	300	300	300	300
5 ul equiv.	2.5	1.25	.625	.3125	.15625

    - Remember to leave a row of cells uninfected for:
      - Count
      - FACS Negative control

- **DAY3:**
  - **Add 0.5 mL of fresh growth medium** on the following day.

- **DAY5 (72 hours post infection):**
  - **Analyze the plates by**
    - **Microscopy (observe in PBS)**
    - **Flow Cytometry**
      - Remove PBS and add ~300µL of trypsin
      - Pipet vigorously after a few minutes, strain through filter, and analyze on flow cytometer. (no need to rinse the cells)
      - Determine Effective Transduction volume [1]

Viral Titer: Calculating ECTV[1]

From Modelling breast cancer requires identification and correction of a critical cell lineage-dependent transduction bias. Nat Commun, 6, 6927:

Calculate ECTV by determining the volume of inoculum needed to infect 50% of cells under a *specific* set of experimental conditions, (Vol_{50}):

$$ECTV = \frac{Vol_{50}}{(N)} * \frac{1}{0.693}$$

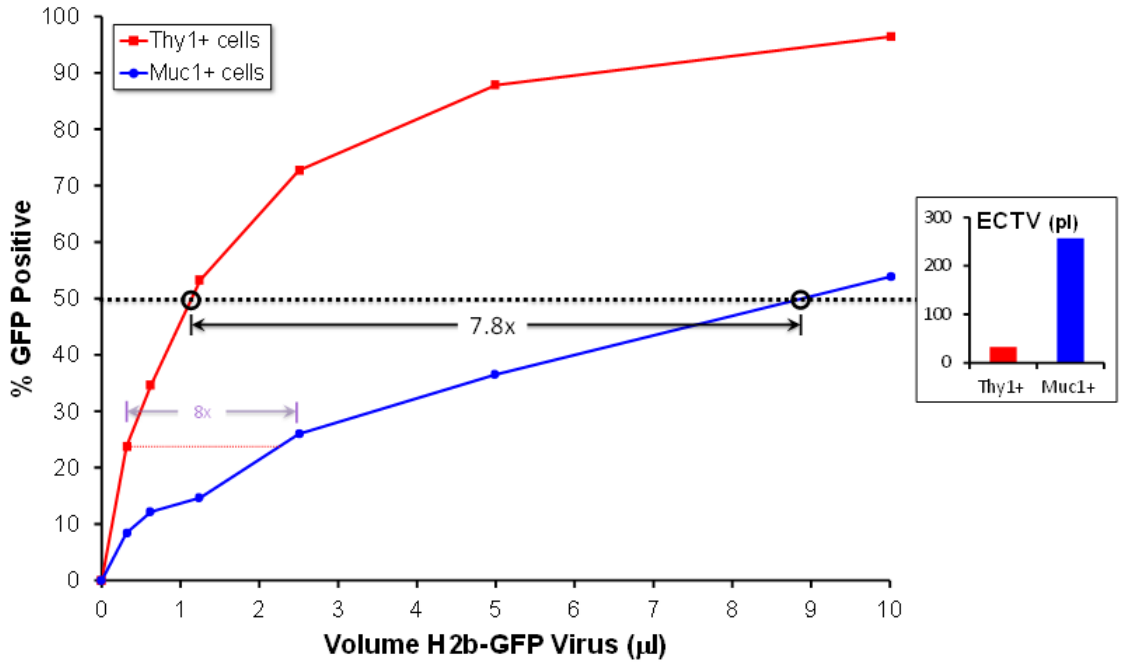
For cases when the fraction of infected cells does not approach 50%, calculate ECTV by:

$$ECTV = \frac{Vol_{X\%}}{-N(\ln(1 - \frac{x\%}{100}))}$$

Once ECTV is determined for the type of cell, virus preparation and set of conditions, the volume of virus needed to infect a specific fraction of cells in subsequent experiments, **using identical experimental conditions** is given by:

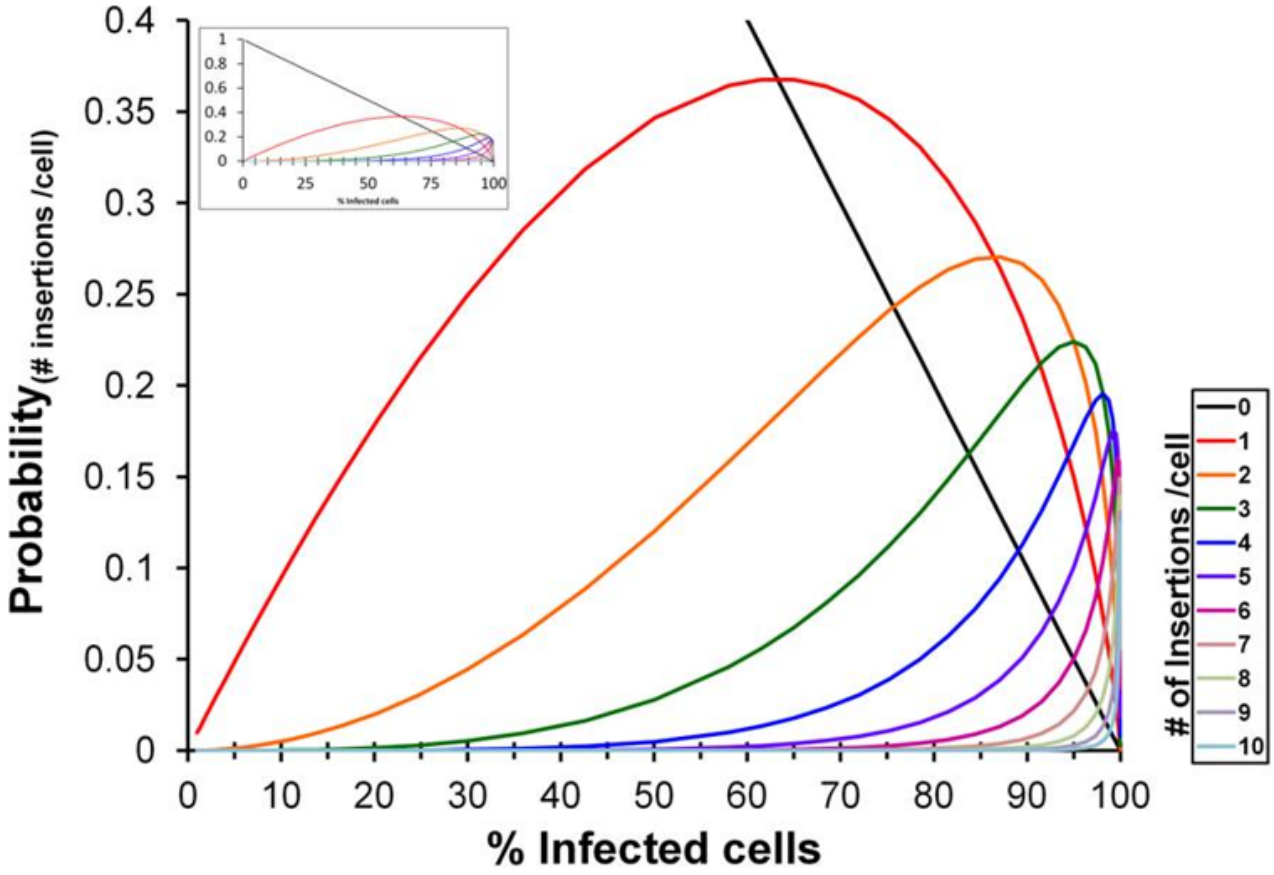
$$Vol_{y\%} = -ECTV(N)\ln(1 - \frac{y\%}{100})$$

Supplementary Figure 4



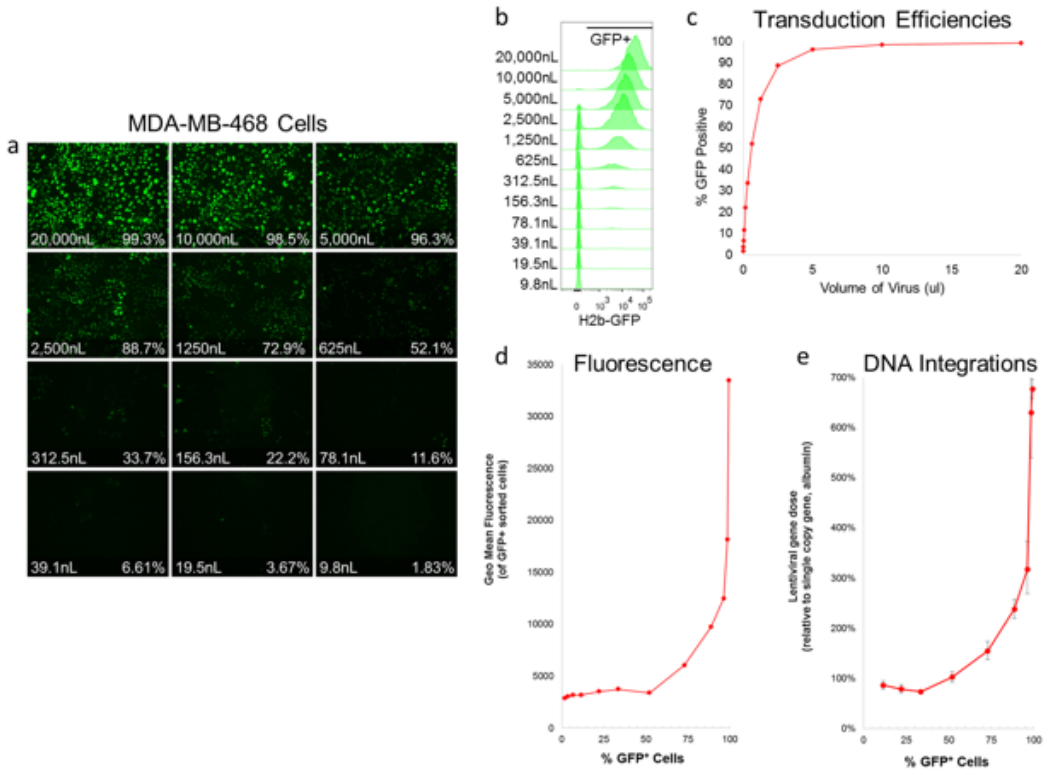
Measuring transductional bias through ECTVs. ECTV values for the transduced primary MEP and LEP subpopulations, shown in Figure 1g, were calculated to be 32.9 and 256.5 picoliters, respectively (*inset, on right*). These values were ascertained using the equation given in Supplementary Note 1 by first determining the amount of virus required by each cell type to achieve a 50% transduction efficiency (dotted horizontal line); i.e. 1.14 µl (MEPs, red) and 8.88µl (LEPs, blue). The ratio of these ECTVs, 7.8x, is equivalent to the fold difference in viral volumes required to obtain equal level of transduction for the MEPs and LEPs, which can be also calculated at different levels of transduction; e.g., at 24% –the transduction efficiency obtained by MEPs at the lowest viral dilution (dotted red line). Each viral dilution is 2-fold, every third is 8-fold (*purple arrows*).

Supplementary Figure 5



Transduction efficiency and acquisition of multiple viral integrations, I. The Poisson distribution is useful in predicting viral transductions (supplementary note 2). Illustrated here is the theoretical relationship between the probable fractions of cells containing 0-10 integration events-per-cell (black, red, orange... traces) to the overall transduction efficiency (percentage of cells with at least one integration event; e.g., GFP^+). For example, when 50% of cells are transduced, the predicted fraction of cells with either 1, 2, or 3 integrations per cell are respectively 35, 12, and 3%, which is 70% (35/50), 24% (12/50) and 6% (3/50) of the ***transduced*** cell population. Inset: graph with y-axis expanded to 100%.

Supplementary Figure 6



Transduction efficiency and acquisition of multiple viral integrations, II. (a) To explore the relationship between transduction efficiency and lentiviral integrations (explained in part I, immediately above), MDA-MB-468 cells were transduced with 2-fold serial dilutions of CMV-H2b-GFP lentivirus and imaged by fluorescence microscopy; volumes of virus and the fractions of transduced cells that resulted from each infection are indicated. (b) Flow cytometry histograms, showing the level and distribution of GFP^+ cells (c) Graph of transduction efficiencies. (d,e) Fraction of cells (% GFP) that became transduced at each viral dose compared to (d) GFP fluorescence and (e) average lentiviral integration events. Lentiviral integrations were measured by qPCR and values are normalized to the single copy gene, albumin, (median±SD, n=4).

1. Hines, W.C., Yaswen, P. & Bissell, M.J. Modelling breast cancer requires identification and correction of a critical cell lineage-dependent transduction bias. *Nature communications* **6**, 6927 (2015).