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SOMATIC CELL GENETIC ANALYSES TO IDENTIFY HIV-1 HOST RESTRICTION FACTORS

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Summary

Cellular proteins are critically involved in all steps of the human immunodeficiency virus type 1 (HIV-1) life cycle. Disruption of host functions essential for virus replication or discovery of new proteins that block viral replication may provide novel antiviral approaches. In recent years, genetic selections for and against genes carried by retroviral vectors have become increasingly powerful, allowing for the isolation of cells with altered susceptibility to virus infection. Screens of complementary DNA (cDNA) libraries, for clones able to induce resistance to infection by recombinant human immunodeficiency virus 1 (HIV-1) genomes, has proved to be an excellent tool to identify new interfering factors. The restriction factors TRIM5 (1), the Zinc Finger Antiviral Protein (ZAP) (2) as well as the dominant negative factor N-86-HnRNPU (3) have all been discovered by means of such genetic screens. Here we report the strategy and techniques to prepare a library and isolate HIV antiviral genes, using the identification of N-86-HnRNPU as an example.

Keywords

HIV-1; cDNA libraries; Genetic Screen; restriction factors

1. Introduction

The strategy developed to select and recover antiviral genes from a library involves: expression of a cDNA library in cells highly susceptible to viral infection; the efficient transduction of these cells with a virus carrying a selectable genetic marker; the elimination of the infected cells using a drug targeting the genetic marker; and the isolation and recovery of the resistant clones. In this chapter we describe each of these steps. The methodology was actually used for the identification of N-86-HnRNPU. In our example described here, a cDNA expression library was constructed using the MLVbased vector pBabe-HAZ (2). The cDNAs were expressed as fusion proteins with a hemagglutinin (HA) epitope tag at the 5' end and the zeocin resistance marker (Zeo) at the 3' end. The retroviral vector contains a LoxP site in the 3' LTR, which after reverse transcription becomes duplicated onto both LTRs such that the provirus can be excised from the genome by the Cre recombinase. The cDNA library was introduced into the human rhabdomyosarcoma cell line, TE671, highly susceptible to HIV-1 infection. Pools of the transduced cells were selected for Zeocin resistance, ensuring expression of at least one member of the cDNA library per cell. To recover any HIV-resistant clones, pools of the transduced TE671 cells were repeatedly challenged with VSV-G pseudotyped HIV-1 retroviral particles expressing the Herpes

Simplex virus (HSV) thymidine kinase (*TK*) gene, to infect as many susceptible cells as possible. The virus-susceptible cells, having become HSV-TK+, were then eliminated by growth in media containing Ganciclovir (GCV), and the rare HSV-TK-negative clones were recovered as potential HIV-resistant clones. We will also describe the methods to retest and confirm the ability of the recovered cDNA to confer virus resistance.

2. Materials

2.1. Tissue Culture

1. Eukaryotic cells grown at 37°C in a humidified atmosphere containing 5% CO₂.
2. DMEM: Dulbecco's modified eagle's medium (Gibco)
3. FBS: Fetal Bovine Serum (Atlanta)
4. L-Gln: 200 mM L-glutamine solution in saline (Gibco)
5. Penicillin: solution at 10,000 U/mL (Gibco)
6. Streptomycin: 10,000 µg/mL Streptomycin sulfate in 0.85% saline (Gibco-BRL)
7. TE671 (ATCC HTB-139) and 293T fibroblast cells were obtained from the American Type Culture Collection.

2.2. Transfections

1. Fugene 6 (Roche Molecular Biochemical's)
2. FBS free OptiMEM (Gibco)

2.3. Retroviral vectors and plasmids

1. pBabe-HAZ: MLV based retroviral vector (2) modified to SalI-NotI (3)
2. pMD.G: VSV-G envelope protein (4).
3. p8.91: HIV Gag-Pol expression vector (4).
4. pHit60: MLV Gag-Pol expression vector (5).
5. pCre: Cre recombinase expression vector (2).

2.4. cDNA library construction

1. A 16°C and 37°C water bath
2. RNA extraction kits, Rneasy Midi kit (Qiagen)
3. mRNA purification kit, Oligotex kit (Qiagen)
4. RNase free -mercaptoethanol
5. RNase free water
6. RNase free Ethanol 100% and 70% (v/v)
7. RNase free NH₄Ac 7.5M (filtered though a sterile 0.2µm nitrocellulose filter)
8. 1 to 10 µl [-³²P]dCTP (1µCi/µl)
9. Phenol:chloroform:isoamyl alcohol (25:24:1)
10. Electrocompetent cells, Stbl2 (Invitrogen)
11. Restriction Enzymes: Sal I and Not I

2.5 Production and titrations of VLPs

1. Fugene-6 (Roche)
2. TaqMan PCR Mix (Invitrogen)
3. Zeocin (Invitrogen)

2.6. Genetic screen

1. Ganciclovir (Sigma)
2. Zeocin (Invitrogen)

2.7. Recovery of cDNA insert

1. DNeasy Tissue Kit (Qiagen)
2. High Fidelity kit (Roche)

2.8 Strategies to retest cDNA ability to confer resistance to HIV infection

1. G418 (Sigma)
2. Puromycin (Sigma)

3. Methods

3.1. Preparation of cDNA library in pBabe-HAZ

cDNA libraries can be directional (oriented for sense-strand expression) or random. All members of a directional library are usually constructed to drive expression of the cloned gene, by a controllable promoter contributed by the vector. Unlike random libraries, in which only 50% of the clones are likely to contain cDNA inserts oriented properly for expression, all members of a directional library are potentially able to express the insert and therefore, a given cDNA can be recovered by screening half as many clones. We will be using as an example a replication-defective retroviral vector based on the Moloney Murine Leukemia Virus (MMLV) genome, designated pBabe-HAZ, that expresses the cDNA inserts under the control of a constitutive promoter (Figure 1). This vector allows expression of genes, or portions of genes, as fusion proteins with the zeocin resistance marker (Zeo), and has several advantages: 1) the ability to be packaged using a variety of envelope proteins, allowing delivery to a wide range of cell types; 2) the ability to allow easy recovery of introduced genes from transduced cells; 3) the presence of LoxP sites such that the cDNA can be excised for assessment of its functionality and 4) expression of a hemagglutinin (HA) tag on the fusion protein to facilitate biochemical studies.

3.1.1. mRNA preparation—The first step in cDNA construction is the isolation of the cellular mRNA. RNA is extremely sensitive to RNases and should be prepared with care. Very small amounts of RNases are sufficient to degrade RNA. The most common sources of RNases are from hands and dust particles. Therefore extra caution is needed for the preparation and storage of RNA. The tubes, tips, and water should be RNase free and gloves should be worn all times.

1. Count 50×10^6 cells of the desired cell type you wish to make the cDNA library from and wash once in PBS 1×. The cells should be sub confluent and growing in log phase.
2. Extract RNA according to the manufacturer's protocol (RNeasy Midi Kit, Qiagen):
 1. Use 4ml of RLT buffer (lysis buffer) with 10 μ l β -mercaptoethanol/ml

2. Homogenize cells by passing the cells through a 19 G needle at least 20 times.
3. Elute twice in 250 µl of RNase free water.
4. Use 5 µl of total RNA in 995 µl of RNase free H₂O to determine concentration by optical density (OD) at 260nm.
3. Purify mRNA from total RNA using Oligotex kit (Qiagen).
 1. Adjust volume of total RNA to 500 µl.
 2. Use 55 µl of Oligotex suspension beads.
 3. Elute twice with 30 µl of elution buffer (OEB). Pool fractions.
 4. Run 3 µl on a gel to check the quality of the RNAs. (See Note ^{#1}). Keep mRNA at – 80°C.
4. Precipitate mRNA and resuspend as follows:
 1. Mix: 50 µl volume of mRNA
 - 5 µl 7.5M NH₄Ac (1/10 volume)
 - 110 µl ethanol 100% (2 volumes)
 2. Incubate for 30 min at 4°C to allow precipitation
 3. Centrifuge at maximum (max) speed for 30 min
 4. Resuspend in 200 µl of Ethanol 70%
 5. Centrifuge at max speed for 30 min to pellet mRNA
 6. Remove supernatant and allow pellet to air-dry
 7. Recover in 6 µl RNase free water.
 8. Use 1 µl in 99 µl of RNase free water to determine OD at 260nm.

3.1.2. cDNA synthesis and cDNA cloning—For cDNA synthesis and cloning use Invitrogen's kit "SUPERScript™ Plasmid System GATEWAY™ Technology for cDNA Synthesis and Cloning". We adapted the kit to the following protocol:

3.1.2.1. First Strand synthesis: Start with 5 µg of mRNA (unless you would like to favor the synthesis of short cDNA fragments, and in that case use 15 µg), in a 5 µl final volume.

1. Add 2 µl of Not I primer-adapter
2. Heat to 70°C for 10 minutes, quick chill on ice, and add:
 - 4 µl 5× First Strand Buffer
 - 2 µl 0.1 M DTT
 - 1 µl 10 mM dNTP Mix
3. Leave 2 min at room temperature to equilibrate temperature, and then add:
 - 5 µl of Superscript II RT (200 units/µg of mRNA for 1 to 5 µg of mRNA)
4. Mix gently and incubate for 1 h at 37°C

^{#1}Wash the electrophoresis tank with bleach, and rinse with DEPC water Use DEPC water to make the gel, and RNase free agarose.

5. Place on ice to terminate reaction.

3.1.2.2. Second strand synthesis:

1. On ice add the following reagents in order:
 - 92 µl DEPC water (0.1% diethyl pyrocarbonate in water)
 - 30 µl 5× second strand buffer
 - 3 µl 10mM dNTP Mix
 - 1 µl *E. coli* DNA ligase
 - 4 µl DNA polymerase (10 units/µl)
 - 1 µl RNase H
 - 1 µl [^{-32}P]dCTP (1 µCi/µl)
2. Gently mix and incubate 2h at 16°C
3. Add 2 µl of T4 DNA polymerase and continue incubating for 5 min
4. Place reaction on ice and add 10 µl 0.5 M EDTA
5. Add 150 µl phenol:chloroform:isoamyl:alcohol (25:24:1), vortex, and centrifuge at maximum speed at room temperature for 5 min
6. Remove 140 µl of upper aqueous solution and transfer to 1.5ml microcentrifuge tube.
7. Add 70 µl of 7.5 M NH₄OAc and 0.5 ml absolute ethanol
8. Vortex and centrifuge at room temperature for 5 min at max speed
9. Remove supernatant carefully
10. Overlay the pellet with 0.5 ml 70°C ethanol.
11. Centrifuge for 2 min at 14,000 × g
12. Briefly dry the cDNA pellet to remove residual ethanol (not too long, otherwise it is hard to resuspend).

3.1.2.3. Sal I Adaptor Addition:

1. Add the following reagents on ice:
 - 25 µl DEPC water
 - 10 µl 5× T4 DNA ligase buffer
 - 10 µl Sal I adaptors
 - 5 µl T4 DNA ligase
 - 50 µl final volume
2. Mix gently and allow to incubate overnight at 16°C.
3. Add 50 µl phenol:chlorophorm:isoamyl alcohol (25:24:1), vortex and centrifuge for 5 min at max speed.
 - Carefully remove 45 µl from top layer to a new microcentrifuge tube.
 - Add 25 µl of 7.5M HN₄Ac and 150 µl alcohol 100%

- Vortex and centrifuge for 20 min at maximum speed
- Remove ethanol and let air-dry.
- Resuspend in double the volume mentioned in the protocol:
 - 82 μ l DEPC H₂O
 - 10 μ l RE_{ACT} 3 buffer
 - 8 μ l Not I
 - 100 μ l final volume
- Incubate for 2 h at 37°C
- Precipitate with 100 μ l of phenol:chlorophorm:isoamyl alcohol (25:24:1), vortex and centrifuge at room temperature for 5 min at 14,000g
- Carefully remove 90 μ l of top phase, and transfer it to a new tube.
- Add 50 μ l 7.5M NH₄OAc and 300 μ l of absolute ethanol, vortex and centrifuge for 20 min at max speed.
- Remove supernatant, wash pellet with 500 μ l of ethanol 70%
- Centrifuge at max speed for 5 min
- Remove supernatant and let the pellet air-dry.

3.1.2.4. Column Chromatography: This procedure optimizes size fractionation for larger cDNA fragments and makes cloning of larger insert more probable.

1. Resuspend pellet in 100 μ l TEN buffer [10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 25 mM NaCl; autoclaved].
2. Open the caps of columns and let the buffer go thru. Add 0.8 ml TEN and let it drain completely. Repeat this procedure 4 times.
3. Add 100 μ l of cDNA, and recover 100 μ l into a new microcentrifuge tube.
4. Add 100 μ l of TEN to the column and recover 100 μ l effluent in a second tube. Let the column drain completely and add a new 100 μ l TEN aliquot.
5. Collect single drops in individual tubes continue adding 100 μ l TEN aliquots until you collected 18 tubes with approximately 35 μ l per tube (drop volume).
6. To maximize the range of the cloned inserts you can pool all tubes from the first radioactivity fraction to the fraction with the peak of radioactivity (measure radioactivity with the Geiger counter). Determine the amount of DNA with the spot technique. Note #2.

3.1.2.5. Ligation of cDNA to the vector:

1. Prepare the vector (pBabe-HAZ) DNA by digestion with Sal I and Not I for 2–3 h at 37°C followed by gel isolation. Determine the concentration of the linearized vector.

#2Spot Technique: Start with a 20ng/ μ l dilution of stock DNA ladder. Prepare the following dilutions: 10, 8, 6, 4, 2, 1 ng/ μ l. Prepare a 10 cm culture dish covered with 8 ml of 1% Agarose with 1 μ g/ml ethidium bromide. Dot 1 μ l of each ladder dilution, as well as 1 μ l of your cDNA sample. Measure intensities under the UV and estimate concentration of your samples by comparison with ladder concentrations.

2. You can use more cDNA than the amount suggested by the manufacturer. You should keep an insert to vector DNA ratio of approximately 1:2.
3. Before ligation, precipitate the desired amounts of vector and insert together using:
 - ½ vol of absolute ethanol
 - 2 vol of 7.5 M NH₄OAc
 - 1 µl glycogen (to better see the pellet)
4. Vortex and centrifuge for 30 min at room temperature at maximum speed.
5. Add 150 µl of 70% ethanol at room temperature for 2 min.
6. Centrifuge for 5 min at max speed, air-dry the pellet and resuspend in 8 µl of water
7. For ligation, use the rapid ligation kit from Roche. Add:
 - 2 µl DNA dilution buffer 5×
 - 10 µl T4 DNA ligase buffer
 - 1 µl T4 ligase
8. Incubate at 37°C for 2 min
9. Leave at room temperature for 5 min and then add 20 µl of phenol chloroform
10. Centrifuge at maximum speed for 3 min, and remove top layer to a new tube, then precipitate by adding:
 - 10 µl 7.5M NH₄OAc
 - 60 µl of absolute ethanol
11. Vortex and spin at maximum speed for 20 min.
12. Remove supernatant and wash pellet with 200 µl of 70% ethanol.
13. Centrifuge at maximum speed for 2 min, air-dry the pellet and resuspend in 5 µl of H₂O.

3.1.2.6. Introduction of Ligated cDNA into *E. coli* by Electroporation: For the electroporation, use competent cells such as Stbl2 (Invitrogen). These cells are suitable for stable cloning of direct repeat and retroviral sequences.

1. Prepare 3 tubes each with 30 µl of Stbl2 suspension and 1.5 µl of ligation product.
2. Add each mixture to 3 independent electroporation cuvettes and electroporate at 1.8 KV, 25 µF and 200 .
3. Immediately add 1ml of S.O.C. (Super Optimal Catabolite) medium to the electroporated cells, transfer the mixture to 14 ml round bottom tubes and incubate 1 h at 37°C with vigorous aeration.
4. Plate 150 µl of cells per 15cm LB plate containing 100 µg/ml ampicillin. Use approximately 20 plates per library. Prepare a test plate by diluting 1 µl of cells in 1ml of S.O.C. and plating 200 µl. NOTE #³.

^{#3}Alternatively, incubate test plate overnight at 37°C and store the remaining transformed cells at 4°C overnight. Count the colonies on the plate, calculate the number of colonies that would result from plating the entire 1 ml of cells, and plate them at a density suitable for screening. Some loss of viability may occur upon storage. If the number of colonies obtained is not sufficient for your needs, you can electroporate an additional 1.5µl of ligation mixture and repeat the procedure.

5. Incubate overnight, count the colonies on the test plate, and calculate the number of colonies that would result from plating the entire 1 ml of cells. This is the number of colonies per μl of electroporated cells resuspend in 1 ml of S.O.C (call it Y). To determine the number of bacterial colonies in your library: multiply Y by 150 (which is the volume plated per plate) by the number of plates used to plate the entire library. This will define the complexity of the library.
6. Pick 10 colonies from the plate as samples for examining inserts, and grow them in 4 ml LB with 100 $\mu\text{g/ml}$ ampicillin overnight.
7. To recover pools of the colonies, add 10 ml of LB to the plates with the transformed cells. Shake very slowly for 15 min to allow cells to detach from agar and scrape the cells.
8. Preserve the transformed cells by preparing 10 vials glycerol stocks: add 750 μl of transformed cells to 250 μl of 50% glycerol mix and store at -80°C .
9. Recover plasmid DNA from remaining cells using a Maxiprep kit (Qiagen). Determine concentration at 260nm.
10. Recover plasmid DNA from the 10 minicultures grown overnight with Miniprep kit (Qiagen), digest the DNA with the enzymes Sal I and Not I, run the digested product in a 1% agarose gel to determine the range of the inserted fragments.

3.2. Production of Virus Like Particles (VLP)

To produce a library of transducing viruses, the cDNA library cloned into a retroviral vector is transfected along with a Gag-Pol expression vector (Gag and Pol proteins corresponding to the same virus as the backbone of the retroviral vector used for the cDNA library construction) and the pantropic VSV-G *env* gene from the Vesicular Stomatitis Virus. The mixture of the 3 DNAs is transfected into 293T cells in a 10 cm culture dish at approximately 70% confluence with Fugene (Roche). Other transfection methods may be utilized as well. In this protocol, transfect the cDNA library cloned in pBabe-HAZ as follows:

1. Mix in a total of 15 μl of TE the plasmids:
 - 2 μg of cDNA library
 - 1 μg of pHit60
 - 1 μg of pMD.G
2. Prepare 200 μl of OptiMEM without serum or antibiotics and add 18 μl of fugene. Do not allow Fugene to touch the sides of the tube; mix by flicking.
3. Add the DNA mixture
4. Incubate for 20 minutes at room temperature
5. Change the media of the cells with 8 ml of complete media (DMEM, 10%FCS, Penicillin/Streptomycin).
6. Add the DNA mix to the cells drop wise, and swirl the plate. Return to incubator.
7. Change media next day, but it is not essential. Note ^{#4}.

^{#4}If you need to prepare VLPs with no viral DNA contamination, you should change the media, and moreover you should treat the collected virus with DNase prior to infection. For this add 75 U of DNase per ml of virus in the presence of 10mM MgCl_2 for 1h at 37°C .

8. Collect supernatant at 48h, 72h and 96hs post transfection 48h post transfection. The culture supernatant can be collected at. At each collection replace the media with fresh 8 ml complete media.
9. Filter with a 0.45µm filter, aliquot and store at –80°C. Avoid freeze thaw cycles, as infection efficiency decreases. Add Hepes pH 7.5 to 50 mM final concentration.

Any type of VLP, not just cDNA carrying VLPs, can be produced in the same manner; for example for the preparation of VSV-HIV-TK, or VSV-HIV-Puro, the 2µg of cDNA library would be replaced respectively by an HIV retroviral vector expressing thymidine kinase (pHIV-TK) or the puromycin resistance gene (pHIV-Puro) and the structural vector should encode HIV Gag and Pol (p8.91) (3).

3.2.1. Titration of VLPs—Depending on the type of VLP produced, different titration methods can be used. If the retroviral vector encodes a resistance gene, you can take advantage of that to titer your viral supernatant. If no resistance marker is expressed, the titer of your virus can be determined by real time PCR, measurement of the viral DNA made after infection.

When determining the titer of a VLP cDNA expression library, titration by drug selection is preferred because it will give you the number of functional cDNAs in your expression library. When titrating by real time PCR you should keep in mind that by measuring the number of integrated proviruses, you are not determining the precise number of cDNAs that will actually be expressed as protein in the cell.

3.2.1.1. Titration by drug selection:

1. Day 1: Plate HIV sensitive cells (for example, TE671 cells), at 1×10^5 cell per 6 well plate.
2. Day 2: Next day, infect the cells with serial 10 fold dilutions of the virus to be titered according to the table below:

Well	1	2	3	4	5	6
Virus (µL)	100	10	1	0.1	0.01	0
Cell #	10^5	10^5	10^5	10^5	10^5	10^5
Dilution:	200 µl virus	200µl dil.1	200µl dil.2	200µl dil.3	200µl dil.4	media
	1.8ml media *	1.8ml media	1.8ml media	1.8ml media	1.8ml media	

* The complete media used may contain 1/1000 dilution of Polybrene (stock 5mg/ml). Polybrene increases the efficiency of the VSV-G envelope entry into the target cells. In some cell types, however, polybrene might actually decrease infection efficiency. Test your cells first.

3. Use 1 ml for each infection (several dilutions are performed, because you do not know at which dilution you will be able to count the number of colonies).
4. Day 4: forty-eight hours post-infection, add the selective drug to the media. Some average concentrations used for human cells, for the most popular drugs are:

Neomycin – 400 µg/ml

Puromycin – 1 to 5 µg/ml

Zeocin – 50 to 100 µg/ml

Hygromycin – 50 µg to 1 mg/ml

However, sensitivities vary with cell line, and therefore you should perform a killing curve on the type of cells you are using.

5. Day 5: Change the media and add fresh media with drug whenever cells start to die. Then keep changing every 3 days. Non-infected cells usually die after about 2 weeks post infection. Note ^{#5}

When visible colonies can be observed, stain the plates with blue Giemsa, and count the colonies as follows:

Empty media and add 100% methanol at -20°C for 10 min room temperature

Add Blue dye (1/20 giemsa: H₂O; 10% methanol) for 1 h at room temperature

Wash plates in large volumes of water several times and allow to dry

Count the colonies

6. The virus titer is equal to the number of colonies on the plate times the dilution factor.

3.2.1.2. Titration of VLP by quantitative PCR:

1. Day 1: Seed sensitive cells (ex: TE671), at 1×10^5 cells per well of 6 well plate.
2. Day 2: Next day infect the cells with serial dilutions of the virus to be titered as described above.
3. Day 3: Remove media, wash cells with $1 \times \text{PBS}$, and add fresh complete media.
4. Day 4: Wash once in $1 \times \text{PBS}$,

Trypsinize cells, wash again in $1 \times \text{PBS}$

Prepare total DNA using DNeasy Kit (Qiagen), following manufacturer's instructions.

Recover DNA in 50 μL elution buffer

Determine DNA concentration by OD at 260nm

Rationale for the standard curves for real time PCR: For example, for the HIV-Puro expressing vector (pSCPW is 9542 nt):

50 $\mu\text{g}/\text{ml}$ of pSCPW is about 4.79×10^{12} molecules/ml.

Prepare serial dilutions of your viral vector, as the following example:

Start with your stock vector at 100 $\text{ng}/\mu\text{L}$, for pSCPW 100 $\text{ng}/\mu\text{L}$ is 9.58×10^9 molec/ml.

10.4 μL of stock in 1000 μL H ₂ O – 10^8 molec/ μ	} Do not take to PCR area
100 μL of 1 in 1000 μL H ₂ O – 10^7 molec/ μL	

^{#5}Some drugs take longer to kill the cells. Puromycin is one of the fastest killing drugs. The day after adding puromycin to cells, usually more than 80 % of the cells die and 100% the next day. Neomycin and zeocin take a little longer, sometimes up to 7 days. You can increase the concentration of the drug if the killing is too slow.

100 µl of 2 in 1000µl H ₂ O – 10 ⁶ molec/µl	}	Use 5 µl per tube for PCR reaction
100 µl of 3 in 1000µl H ₂ O – 10 ⁵ molec/µl		
100 µl of 4 in 1000µl H ₂ O – 10 ⁴ molec/µl		
100 µl of 5 in 1000µl H ₂ O – 10 ³ molec/µl		
100 µl of 6 in 1000µl H ₂ O – 10 ² molec/µl		

Primer pairs and probes that can be used to quantify an MLV or HIV based VLP are summarized below (they encompass the packaging sequence):

Primers	sequence (5' 3')
5'HIV	TGGGCAAGCAGGGAGCTA
3'HIV	TCCTGTCTGAAGGGATGGTTGT
5'MLV	CATGGACACCCAGACCAGG
3'MLV	CGGATGGAGGAAGAGGAGG
Probes	
HIV	[DFAM]ACCGATTGCGAGTTAATCCTGGCCTGTT[DTAM]
MLV	[DFAM]GCCCCCTACATCGTGACCTGGGAAGCC[DTAM]

Mix per reaction (using TaqMan):

1.5 µL of 1/10 dilution of stock primer 5 (50 µM)
1.5 µL of 1/10 dilution of stock primer 3 (50 µM)
0.75 µL of 1/10 dilution of stock probe (5'FAM and 3'TAMRA) (50 µM)
12.5 µL PCR Mix (TaqMan Universal PCR Master Mix, Applied Biosystems)
2.75 µL of water
19 µL

Calculate amounts for number of samples :	x samples
	10 standards
	2 blanks
	+2 (for error)
	y point reactions

Add 19µL per well of PCR plate for standards and blanks

Add 5µL of each standard dilution starting on 10⁵ molecules

Add Carrier to the remaining mixture $(x+2) \times 5\mu\text{L}$ carrier (1/100 dilution of salmon sperm DNA)

Add 24 μL of this final mixture in PCR plate for the x number of samples

Add 2 μL of DNA template

Run PCR program:

95°C - 10 min - 1 cycle	
95°C - 15 sec	} 40 cycles
60°C - 1 min	
4°C - 10 min	

Viral copies should be normalized per total DNA input, and you can define the titer as viral copy number per 100 ng genomic DNA. Otherwise, assuming that 1 cell contains in average a total of 6 pg, determine the number of provirus per cell, and from there, knowing the number of plated cells, and volume used, calculate number of particles per ml of viral supernatant.

3.3. Production of a population of cells transduced with expressed cDNAs cloned into pBabe-HAZ

A single member of the cDNA library should be expressed per cell, to avoid screening clones with several cDNAs, because it would be harder to identify the clone responsible for the resistance. To make sure that one cDNA is expressed per cell, choose an MOI of approximate 0.25, or one virus per 4 cells.

In order to determine the starting cell number to infect with the VLP-cDNA library, a choice has to be made of how many clones to screen at a time. Keep in consideration that if you titered your library by real time PCR, not all of the proviruses measured will actually express any fusion protein. Therefore, estimate the titer of the library to be at least 1/3 less, due to the possible cloning of the cDNA in any of the three reading frames with only one forming a functional zeocin fusion protein. However, if resistance to zeocin was used to measure the titer you have the accurate titer.

When considering the type of eukaryotic cells to use to express the cDNA library, and perform the screen, keep in mind that these cells must have the least possible level of background resistance, not only towards the HIV infection, but also, to the drug used to kill the cells that became infected. A killing curve should therefore be performed on the cells you chose to use, to assess their infectability. Note #6.

#6 The most appropriate cells to perform the screen have to be extremely sensitive to infection, and readily killed by the drug you use to eliminate infected cells. Therefore, test first which cell type has the highest sensitivity to infection. Seed identical amounts of cells of the lines you want to test and infect with 3 different MOIs of an HIV-VLP (for example VSV-HIV-GFP). Assess the percentage of infected cells (by FACS in this example). Choose the cell line for which the lowest amount of virus achieved the maximum amount of infected cells.

Next, determine the amount of drug needed to eliminate infected cells. Prepare 10 plates with 1×10^5 cells per 10cm plate. Infect 5 plates with the challenging virus (in this example VSV-HIV-TK), in order to cover approximately 100% of the cells. Leave the other 5 plates uninfected, to serve as the uninfected control for each concentration of drug to be tested. Forty hours later, prepare 5 different drug concentrations to be tested for elimination of infected cells (in this example GCV). Add each concentration of drug to a pair of plates, one infected and one uninfected. Determine which concentration of drug allowed the fastest killing of the infected cells, without being toxic to uninfected cells. Chose this concentration to perform the genetic screen.

The authors *Gao et al* (2) and *Stremlau et al* (1), used Rat 2 and HeLa cells to identify ZAP and TRIM5 respectively. In this example, we used the rhabdomyosarcoma cell line, TE671 that is highly sensitive to HIV infection. A population of cells expressing the cDNA library was established by selection with zeocin as follows:

1. Set up the number of cells equivalent to four times the number of cDNA clones to be screened. It is preferable to plate the cells in 15cm culture dishes at 30 to 40% confluence. If cells are plated at high density, you will probably need to split the cells very soon after. Lower densities will allow for the selection to start without having to split the cells right away.
2. Next day, in the presence or absence of 0.5 µg/ml of polybrene (according to the cells used), dilute your virus to the appropriate concentration in the minimum volume possible (12ml per plate should be enough) and incubate the virus for at least 4 hours.
3. Add complete media to a total of 15 to 20 ml per plate.
4. Forty hours after infection, add zeocin at 50 µg/ml in complete media.
5. Keep the cells under selection for approximately 2 weeks, changing the media regularly to remove the dead cells.

The selection should be carried in the smallest amount of time, to avoid loss of cDNA representation in the population due to overgrowth of rapidly growing clones over slower ones.

6. Make frozen stocks of this population in 15% DMSO in FCS. Store at -160°C.

3.4. Screening of the library and isolation of clones conferring resistance to HIV infection

The rationale for selecting cDNA fragments capable of conferring resistance to HIV infection is to submit the cDNA expressing cells to several rounds of infection with a genetically marked HIV particle, treat the cells with a drug that will kill the infected cells, and recover the resistant clones that would be possibly resistant due to the presence of the cDNA.

In this example, we used the genetically modified virus VSV-HIV-TK, a retrovirus particle encoding the Herpes Simplex Thymidine Kinase (HSV1-TK) under the control of a constitutive promoter, and pseudotyped with the pantropic VSV-G envelope protein. The virus can be prepared and titered as described in section 3.2.

To eliminate the infected cells we chose to treat cells with Ganciclovir (GCV). GCV is a synthetic nucleoside analog of 2'-deoxy-guanosine that can be enzymatically phosphorylated into an active triphosphate analog by HSV-TK. The phosphorylated product is incorporated into the DNA causing death of the infected cell. GCV may sometimes cause a bystander effect, that is, toxicity in the cells that are in the vicinity of an HIV-TK infected cell. Therefore a killing curve should be performed in the cell type of your choice, to determine the maximum amount of death by the drug without affecting non-infected cells. The rare TK- cells surviving selection in GCV are then recovered as possible virus resistant cells.

A key point of this screen methodology is the amount of challenging VSV-HIV-TK to be used to infect the pools of cDNA expressing cells. The majority of the cellular restriction factors discovered to date, tend to be saturable, meaning that if the cell is infected with too many viruses, eventually all the restriction factor will be bound by the initial virus, and the following incoming virus will not face any restriction. Therefore, if too much challenging

virus is used you may end with no resistant clones, and if you use too little virus you may end with too many clones, most of them false positives.

We have found that several rounds of infection at a moderate MOI, is sufficient to challenge most of the cells, without overcoming any eventual block.

For TE671 cells expressing the cDNA library in pBabeHAZ we use the following method:

1. Day 1: Set up the pool of cDNA-expressing TE671 cells at approximately 25% confluent in 15 cm culture dishes.
2. Day 2: Infect the cells with VSV-HIV-TK at a MOI of about 0.1 in the minimum volume possible (12 ml should be enough per plate). Incubate for 4 hours.
3. Aspirate the media and repeat the infection 2 more times.
4. Day 3: Next day repeat the infections 3 to 4 times.
5. Day 4: Add Ganciclovir at 50 μ M in complete media. The killing by GCV is not immediate; it might take a few days to observe cytotoxicity. When cell death is observed, the culture supernatant should be replaced with new GCV-containing media. In the beginning of the screen you might need to change the selection media every day, and then, when not that many cells are left alive, it is better to leave the media for longer as growth factors will enrich the media and facilitate the growing of individual clones. If you have many cells in the plate, you should split them, and keep the plates at 30 to 40% confluence. GCV treatment is more efficient when cells are actively replicating. Upon a week or two of GVC treatment there are three possible outcomes:
 - a. There are still a lot of cells in the plate after the GCV treatment, most probably background resistance, and therefore another round of infections with VSV-HIV-TK at low MOI is required. Repeat the infection and GCV selection.
 - b. There are no cells left in the plates. Two things could have happened: 1) there are no restricting cDNA fragments in the batch of pooled cDNA-expressing cells tested, or 2) too much of the VSV-HIV-TK challenge virus was used and the block was overcome. In both cases, the screen has to be restarted again with another batch of pooled TE671 cDNA library expressing cells.
 - c. There are a few cells in the plates that are struggling to survive. In this case, add conditioned media to the plates. Use media recovered from plates growing the same type of cells at a good confluence; this media is enriched with growth factors that will help boost growth of single clones. Filter it with 0.45 μ m filter and add to the plates in the presence of GCV. When the clones achieve a visible size, recover the clones and gradually amplify by growing them in culture dishes of increasing size. Be very careful at this stage not to lose any potential resistant clones.

There is a fine line between recovering too many clones that are just false positives, and overcoming the cellular block to the virus and ending with no resistant clones at all. However, if a strong consistent cellular block to the virus is desired a reasonable amount of virus has to be used. This will be the longest part of the process.

There are other strategies to recover cDNA clones resistant to HIV that may be less time consuming. For example, the screen used in the identification of TRIM5 , involved sorting the cells by FACS (Fluorescent Assortment Cell Sorter). The cDNA library expressing cells

were challenged with an HIV vector expressing GFP (green fluorescent protein), VSV-HIV-GFP. Sufficient virus was used to infect at least 99% of the cells. About 0.5% of the cells were selected for absence of fluorescence by FACS. These cells were allowed to grow and then subjected to a second round of VSV-HIV-GFP infection at a higher MOI. GFP negative colonies were identified by fluorescent microscopy, cloned and expanded.

Even though this strategy might be less time consuming, it is subject to the same considerations mentioned above about the amount of virus used to challenge the library. Moreover, the limit of GFP detection of the FACS machine may lead to different outcomes. If the cut off is too low it may result in the exclusion of some not so strong blocks, but potentially interesting (for example blocks in the mRNA export), if the cut off is too high it might lead to increased background. Also, keep in mind that some cell lines are autofluorescent and therefore not appropriate for this methodology.

3.5. Recovery of the cDNA insert in resistant clones

Once the resistant clones have been amplified to about a confluent well of a 6 well plate, use part of these cells to prepare total DNA and recover the cDNA insert by PCR. The rest of the cells should be amplified and frozen vials should be prepared with 15% DMSO in FCS and kept at -160°C .

From each resistant clone recovered:

1. Prepare total genomic DNA using the DNeasy Tissue kit (Qiagen), elute in 100 μl of buffer AE, and repeat the elution using the first eluate to increase final concentration.
2. Prepare PCR mix (using High Fidelity kit, Roche):

1 μg	Total DNA as template
5 μl	5' primer – 5' GCTTATCCATATGATGTTCCAGATT 3'
5 μl	3' Primer – 5' GCACCGGAACGGCACTGGTCAACTT 3'
5 μl	MgCl ₂
5 μl	Buffer 3
4 μl	dNTP
0.5 μl	Taq polymerase (High Fidelity, 1.75 U)
50 μl	Final volume

Run PCR program with the following parameters:

94°C - 10 min – 1 cycle	} 10 cycles
94°C – 15 sec	
50°C – 30 sec	
72°C – 60 sec

Once you recover the PCR product, sequence the DNA fragment to determine the identity of your cDNA insert.

3.6. Strategies to retest the ability of the cDNAs to confer resistance to HIV infection

Once the putative resistance clone is recovered, the functionality of the overexpressed cDNA has to be determined. The resistance to viral infection could be due to the cDNA fusion protein expression, or to somatic mutations incurred during the selection process.

Two approaches can be taken to assess the role of the cDNA in the observed resistance; a) excise the cDNA fragment from the resistant clone and test if the sensitivity to HIV infection is recovered and b) reclone the cDNA fragment onto pBabe-HAZ, express the fusion protein in fresh cells and test if cDNA expressing clones become resistant to infection. These assays will ensure that the cDNA is sufficient and necessary for the observed restriction.

3.6.1. Excision of the cDNA insert—The vector pBabe-HAZ contains a lox P site in the 3'LTR. Upon reverse transcription of the vector, another Lox P site is duplicated into the 5' LTR. These two sites can be used to excise the provirus using the Cre recombinase.

3.6.1.1. Transfection and selection of Cre expressing clones:

1. Transfect 1 µg of the pCre expressing vector along with 25 ng of a selectable marker DNA encoding drug resistance of your choice, other than zeocin^R, into the resistant clones. We used a Neomycin resistance marker.
2. 48 h later, add 400 µg/ml of G418 in complete DMEM.
3. Select for G418 resistant clones. Set up 10 cm plates with dilutions of the resistant population at 1/100, 1/200 and 1/500. Keep the cells under selection, and recover individual clones. Gradually amplify them.
4. Once the G418 resistant clones have been expanded determine if the cDNA fragment has been excised. Extract total DNA from each clone and determine the presence or absence of the cDNA insert by PCR as described in section 3.5.

3.6.1.2. Test Cre-Lox clones for HIV infectivity: To test if the newly established clones have regained their sensitivity to infection, they should be challenged with a HIV-VPL. It is important to use another reporter gene for this assay, to ensure that the observed resistance developed towards virus and not merely against the reporter.

1. Chose 4 or 5 clones in which cDNA excision was successful and seed 1×10^5 cells per 10 cm dish. Prepare 3 dishes per clone. In parallel, prepare 3 plates with 1×10^5 cells of the wild type cells expressing the empty vector, and 3 plates with the initial resistant clones expressing the cDNA insert. These will be, respectively, the positive and negative controls.

2. Next day, infect the cells with another reporter HIV VLP at three different MOIs, for example, VSV-HIV-Puro. The number of puromycin resistant colonies at three different MOIs will help score the sensitivity to infection. When choosing the 3 MOIs to use, keep in mind that 1×10^5 cells were plated and that a maximum of approximately 500 colonies can be counted per 10 cm plate (this figure is dependent on the cell type). Therefore the highest MOI to be used should be around 5×10^{-3} .
3. 48 h later add 2 to 5 μ g/ml of puromycin and follow same protocol as for virus titration, section 3.2.1.a.

If the cDNA insert is responsible for the observed HIV-1 resistance, the number of colonies observed for the excised cDNA clones should approach the number of resistant colonies found on the empty vector control plates. In contrast, the number of colonies determined for the cDNA expressing resistant clones should be much lower, at least 10 times lower, to be worth pursuing its study.

3.6.2. Expression of the cDNA in fresh cells—

1. Amplify the cDNA insert from the genomic DNA as described in section 3.5. Clone this PCR product into the vector pBabe-HAZ by using the restriction sites Sal I/Not I. Package the cDNA into a VLP as described in section 3.2, or else transfect the vectors directly into fresh cells TE671.
2. Select cDNA expressing clones by selecting cells with zeocin and prepare individual clones as described above. Prepare empty vector clones in parallel as controls.
3. Once cDNA expressing clones have been amplified, extract total genomic DNA and determine the presence of cDNA insert by PCR and as in section 3.5.
4. Test the sensitivity to infection in the same way as described in 3.6.1.b. This time, one would expect that the cDNA-expressing clones should yield the same low number of colonies as the original cDNA-expressing clone used as negative control.

3.7. Test replication of wt HIV

It is important to determine if the resistant clones obtained are effective in blocking wild type HIV replication. There is always the possibility that the cDNA targets a part of the HIV-VLP that is not actually present in the wild type particles. We will determine virus replication by measuring reverse transcriptase RT activity.

To test wt HIV replication, the target cells need to express the CD4 and CXCR4 receptors. If the cDNA expressing cells do not express any of these receptors, as in the case of TE671 cells (CXCR4⁺ only) then the gene for the missing receptor has to be introduced, or the resistance inducing cDNA can be expressed in cells that already express the two receptors. One can use adherent or non-adherent cell lines to express the cDNA. Some examples are: HeLa CD4/CXCR4, CEM or Jurkat. Make cellular clones expressing the cDNA fragment and expressing both receptors in any of these ways.

3.7.1. Wt HIV infections—

1. HIV-1 particles can be prepared by transient transfection of 293T cells with the proviral HIV-1 pNL4-3 DNA (6). Virus stocks for infections are produced by amplification of the virus by acutely infecting CEM SS cells with HIV-1 pNL4-3 and concentration by ultracentrifugation of the cell supernatant.

2. To initiate infection:

Day 1: Seed 1×10^5 of the target cells per well in a 6 well plate. Prepare a control plate with the corresponding cell type expressing empty vector lacking the cDNA.

Day 2: Infect the cells. Use serial 10 fold dilutions in complete DMEM of the concentrated virus in 6 well plates, with 1 ml per well of virus dilution. If a non-adherent cell line is used, the cells are seeded and infected on the same day. Count the cells, microcentrifuge, and recover the cell pellets directly in the viral dilutions. Use complete RPMI media for non-adherent cells.

Day 3: Aspirate the media and replace with 1.5 ml of complete DMEM. For non-adherent cell lines, microcentrifuge the cells, discard supernatant and resuspend the cells in 1.5 ml of complete RPMI.

3. From next day on, recover 30 μ l of virus supernatant. Depending on the quality and concentration of the virus used, the cytopathic effects can appear as soon as 24 h after infection or as much as 2 weeks later if very low MOIs were used. Because serial dilutions are performed, it is advisable to start with a highly concentrated stock of virus. Even if there is a risk of overcoming the block with too much virus in the most concentrated wells, differences in viral production at lower MOIs can still be observed.

3.7.2. RT assay—

1. For the exogenous RT assay, load 10 μ l of each of the recovered undiluted cell culture supernatants into each well of a round bottom 96 well plate.
 2. Add 50 μ l of following RT cocktail per point:
 - 60 mM Tris-HCl (pH 8.0)
 - 72 mM NaCl, 12 mM MgCl_2
 - 2 mM dithiothreitol, 0.06% NP40
 - 6 μ g/ml oligo(dT)
 - 12 μ g/ml poly(rA)
 - 0.012 mM dTTP
 - 0.05 μ M [$^{-32}$ P] dTTP (800Ci/mmol)]
 3. Incubate 1h at room temperature.
 4. Spot 5 μ l of reaction mixtures onto DEAE paper cut to replicate a 96 well plate.
 5. Wash 2 times with $2 \times$ SSC (300 mM NaCl, 30 mM sodium citrate (pH 7)) for 20 min.
 6. Rinse the DEAE paper with 95% ethanol and dry under a heat lamp or with an air dryer.
 7. Cover paper with plastic wrap and expose to a phosphorimager cassette overnight.
 8. Use PhosphorImager to scan and quantitate the radioactivity incorporated.
- Plot RT activity versus days post infection. If a true resistant clone was discovered, a drastic reduction in viral production or a significant delay of viral replication should be observed.

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Figure 1. Schematic representation of the pBabe-HAZ construct. Y, MuMLV viral RNA packaging signal; SalI-NotI, linker sequence containing SalI and NotI sites; LoxP, LoxP sequence for recognition by Cre recombinase.