

PROTOCOL FOR CONSTRUCTION OF HIV-1 FUNCTIONAL ENVELOPE CLONES FROM PLASMA

1. Amplification and cloning of full-length HIV-1 envelopes (gp160) from plasma¹

RNA extraction

RNA is extracted from 1-2 ml plasma using MiniMag Nuclisens® (Biomerieux) following manufacturer's instructions, with a final elution volume of 25 µl.

(If EasyMag® is used, start with 1 ml plasma and use final elution volume of 40 µl).

Store RNA frozen at -80°C.

RT-PCR

Primers' sequences and HXB2 positions:

Forward (4809 - 4841)

SG3-up: 5'-TACAGTGCAGGGGAAAGAATAATAGACATAATA-3'

Reverse (9523 - 9544):

SG3-lo: 5'-AGACCCAGTACAGGCRARAAGC-3'

Reaction mix for RT-PCR

<u>RT-PCR:</u>	<u>[stock]</u>	<u>µl per sample</u>	<u>x (µl)</u>
DEPC water		10.82	
10X buffer (Provided with BioTaq)	10 X	2	
MgCl ₂ (Provided with BioTaq)	50 mM	0.8	
SG3-up primer	20 µM	0.4	
SG3-lo primer	20 µM	0.4	
dNTP mix (GE Healthcare Life Sciences)	20 mM each	0.2	
BioTaq DNA polymerase (Bioline)	5 U/µl	0.2	
RNasin RNase inhibitor (Promega)	40 U/µl	0.1	
Pfu Turbo DNA Polymerase (Stratagene)	2.5 U/µl	0.04	
Super Script III Reverse Transcriptase (Invitrogen)	200 U/µl	0.04	
Total premix		15	
RNA		5	
Final volume		20	

Note: In case that single genome amplification is performed, serial two-fold RNA dilutions should be done, assaying 8 to 16 replicates per dilution in 96-well plates to obtain amplified products in less than 30% replicates, checking for the absence of mixed peaks in sequence electropherograms.²

Thermal profile:

50°C 1 h

94°C 2 min

35 cycles:

94°C 15 sec

55°C 30 sec

72°C 5 min

72°C 10 min

4°C 10 min

12°C (store)

Nested PCR

Primers' sequences and HXB2 positions:

Forward (5957 - 5983):

Env-up*: 5'-GTTTCTTTTAGGCATCTCCTATGGCAGGAAGAAG-3'

Reverse (9063 - 9088)

Env-lo*: 5'-GTTTCTTCCAGTCCCCCCTTTTCTTTTAAAAAG-3'

*GTTTCTT is appended to the 5' ends to increase addition of A by Taq polymerase.

Reaction mix for nested PCR

<u>Nested PCR</u>	<u>[stock]</u>	<u>µl per sample</u>	<u>x (µl)</u>
DEPC water		17.95	
10X buffer (Provided with BioTaq)	10 X	2.5	
MgCl ₂ (Provided with BioTaq)	50 mM	1	
Env-up primer	20 µM	0.5	
Env-lo primer	20 µM	0.5	
dNTP mix (GE Healthcare Life Sciences)	20 mM each	0.25	
BioTaq DNA polymerase (Bioline)	5 U/µl	0.25	
Pfu Turbo DNA Polymerase (Stratagene)	2.5 U/µl	0.05	
<u>Total premix</u>		23	
RT-PCR product		2	
Final volume		25	

Thermal profile:

94°C 2 min

30 cycles:

94°C 15 sec

56°C 30 sec

72°C 3 min

72°C 7 min

4°C 10 min

12°C (store)

Agarose gel electrophoresis

Check PCR amplification in a 1% agarose gel with GelRed (1:20,000) (Biotium), loading 5 µl of each nested PCR product (mixed with 1 µl of 6X loading buffer).

Samples that have amplified (~3 kb) will be purified for cloning.

DNA purification for cloning

Prepare 1% agarose gel in 1X TAE buffer with SYBR Safe (1:10,000). Load 14 µl of nested PCR product, perform electrophoresis and do not expose DNA to UV: orange bands are visualized under visible light when large amounts of DNA are present in bands. Cut bands and purify DNA with MinElute GEL Extraction kit (Qiagen) following the manufacturer's protocol, using a final elution volume of 10 µl.

Check recovery by electrophoresing 2 µl of each sample in another 1% agarose gel.

TA cloning

Ligation

Use pcDNA3.1/V5-His©TOPO© TA Expression kit (Invitrogen)

Reaction mix:

2 µl purified DNA (extracted from agarose gel)

1 µl TOPO TA expression vector

1 µl salt solution (provided with the kit)

2 µl sterile water

Incubate 30 min at room temperature.

Transformation

Use MAX Efficiency® *Stbl2*[™] competent cells (Invitrogen)

Pipette 4 µl ligation product in 50 µl *Stbl 2* cells.

For positive control, use 1 µl pUC19 in 50 µl *Stbl 2* cells.

Incubate on ice for 30 min.

Heat shock at 42°C for 25 secs.

Incubate on ice for 2 min.

Add 250 µl SOC medium (previously at room temperature)

Incubate at 30°C for 2h in an orbital shaker at 225 rpm.

Seed transformed bacteria in LB-Amp (100 µg ampicillin /ml LB medium) agar plates:

- 200 µl of each sample.
- 40 µl of pUC19 in a LB-Amp agar plate (positive control).

Incubate at 30°C overnight.

Colony Screening

Screening for bacterial colonies containing the insert in the correct orientation is performed by PCR. For this, colonies are picked with a pipette tip and transferred to a new LB-agar-ampicillin plate, incubating overnight at 30°C. The newly grown colonies are picked with a pipette tip and rinsed in 25 µl DEPC water in a 96-well V-bottom plate. Bacteria are lysed by heating the plate at 96°C for 10 min.

Primers used for PCR are T7 (forward), recognizing a sequence in the vector, and env-lo (reverse), recognizing the 3' end of the cloned envelope fragment. Only colonies with clones in the correct orientation will produce a PCR product ~3 kb.

Reaction mix for colony screening PCR

PCR	[stock]	µl per sample	x (µl)
DEPC water		8.4	
10X buffer (Provided with BioTaq)	10 X	2	
MgCl ₂ (Provided with BioTaq)	50 mM	0.8	
T7 primer (provided with pcDNA3.1/V5-Hi TOPO TA Expression kit)	20 µM	0.2	
Env-lo primer	20 µM	0.2	
dNTP mix (GE Healthcare Life Sciences)	20 mM	0.2	
BioTaq DNA polymerase (Bioline)	5 U/µl	0.2	
<u>Total premix</u>		12	
Lysed bacteria		8	
Final volume		20	

Thermal profile:

94°C 2 min

35 cycles:

94°C 30 sec

55°C 30 sec

72°C 3 min

72°C 10 min

4°C 10 min

12°C (store)

Plasmid prep

Use QIAprep® Miniprep (Qiagen), following manufacturer's protocol.

Typical recovery yield is 300 to 500 ng/µl.

2. Pseudovirus production and envelope functional assay^{1,3}

Day 1:

In 24-well plates, seed 6×10^4 293T cells/well, with 500 μ l DMEM medium (containing 10% FCS and supplemented with 1% penicillin-streptomycin) per well.

Day 3:

Co-transfect 300 ng plasmid containing the cloned envelope and 300 ng pSG3 Δ env* with polyethylenimine (JetPEI™, PolyPlus-transfection) at a 4:1 ratio (2.4 μ l JetPEI™ to transfect 600 ng plasmid DNA):

Prepare two premixes:

Premix A (per sample):

50 μ l NaCl (150 mM)

1 μ l pSG3 Δ env (300 ng/ μ l)

Premix B (per sample):

50 μ l NaCl (150 mM)

2.4 μ l JetPEI™

Vortex each premix and distribute in a sterile 96 well plate as follows:

50 μ l premix A in wells of row A

50 μ l premix B in wells of row B

Add 300 ng plasmid DNA containing each cloned env to the corresponding well containing premix A.

Mix up & down wells in row A with a multichannel pipette.

Transfer premix B (from row B) to row A with a multichannel pipette. Mix up & down with the pipette.

Incubate A+B premix for 30 minutes at room temperature.

Meanwhile, change medium in the 24-well plate seeded with 293T cells. Remove medium and add 0.5 ml complete DMEM medium.

Transfer 100 μ l of A+B premix from the 96-well plate to the 293T cells in the 24-well plate. Pipet-Lite Adjustable Spacer LA6-300 is appropriate to perform this transfer.

**pSG3 Δ env is an env-deficient HIV-1 molecular clone, available at NIH AIDS Reagents Repository and at NIBSC.*

Day 4:

To increase pseudovirus concentration, remove medium and add 220 μ l of complete DMEM medium.

Day 5:

Seed TZM-bl cells in a sterile 96-well flat bottom plate (5000 cells/well in 50 μ l complete DMEM medium).

Collect supernatant containing pseudoviruses from each well of the 24-well plate with transfected 293T cells. Transfer supernatants to 1.5 ml Eppendorf tubes. Spin down the tubes in a microfuge at 1000 rpm for 10 minutes.

Transfer 50 μ l of cell-free supernatant in the corresponding well seeded with TZM-bl cells. This step should be done in quadruplicate. For this, 200 μ l of each supernatant are transferred to an empty well of a 96-well plate and then 50 μ l are distributed with a multichannel pipette to each TZM-bl-seeded well.

Day 8:

Prepare fresh 1X Luciferase Cell Culture Lysis buffer (Promega, cat# E1531) by diluting 5X stock (stored at -20°C) with water.

Thaw Luciferase Assay Reagent (Promega, cat# E1483) (stored at -80°C) at room temperature.

Remove media from the 96-well plate seeded with TZM-bl cells infected with pseudoviruses.

Wash cells with 1X PBS (100 μ l per well).

After removing PBS, add 25 μ l of 1X Luciferase Cell Culture Lysis buffer to each well. Incubate for at least 2 min at room temperature.

Lyse cells up & down with a multichannel pipette.

Transfer 10 μ l lysate to a white OptiPlate™-96 (PerkinElmer).

Add 50 μ l Luciferase Assay Reagent to each well and mix up & down with a multichannel pipette.

Measure luminescence with a Wallac 1420 Multilabel Counter (Perkin Elmer). Count cps during 10 seconds per well.

Functional clones suitable for neutralization assays should yield luminescence values of at least 50 times above background signal.

3. Making a glycerol stock

Glycerol stocks can be made of bacteria with functional envelope clones.

- Pick a colony off the plate and grow overnight in 5-6 ml LB-Amp medium at 37°C, 225 rpm.
- In a cryotube, mix by pipetting 828 μ l bacterial culture with 172 μ l 87% glycerol (15% glycerol final concentration).
- Freeze glycerol stock on a dry ice/ethanol bath and store at -80°C.

References

1. Revilla A, Delgado E, Christian EC, Dalrymple J, Vega Y, Carrera C, González-Galeano M, Ocampo A, de Castro RO, Lezaun MJ, Rodríguez R, Mariño A, Ordóñez P, Cilla G, Cisterna R, Santamaría JM, Prieto S, Rakhmanova A, Vinogradova A, Ríos M, Pérez-Álvarez L, Nájera R, Montefiori DC, Seaman MS, Thomson MM. Construction and phenotypic characterization of HIV type 1 functional envelope clones of subtypes G and F. *AIDS Res Hum Retroviruses* (2011); 27:889-901.
2. Salazar-Gonzalez JF, Bailes E, Pham KT, Salazar MG, Guffey MB, Keele BF, Derdeyn CA, Farmer P, Hunter E, Allen S, Manigart O, Mulenga J, Anderson JA, Swanstrom R, Haynes BF, Athreya GS, Korber BT, Sharp PM, Shaw GM, Hahn BH. Deciphering human immunodeficiency virus type 1 transmission and early envelope diversification by single-genome amplification and sequencing. *J Virol* (2008); 82:3952-3970.
3. Montefiori DC. Evaluating neutralizing antibodies against HIV, SIV, and SHIV in luciferase reporter gene assays. *Curr Protocol Immunol* (2004); Chapter 12; Unit 12.11.