Neutralization assay

Istitute de Salud Carlos III, Spain

Neutnet code: 12
Contact person: Jose Alcami; ppalcami@isciii.es

Viral RNA isolation and amplification of *env* gene

Summary: Viral RNA is extracted using QIAamp® Viral RNA Mini Kit (Qiagen), according to the manufacturers' protocol. RT-PCR is performed with the SuperScript™ One-Step RT-PCR with Platinum® Taq System (Invitrogen, Carlsbad, California) using the primers 30eu and 11ed (Table I). PCR product is used as a template for nested PCR amplification with the Expand High Fidelity PCR System (Roche) and the primers 53eu and 52ed containing XbaI and NotI sites respectively (Table II). The resulting 2.7 kb amplification products include the whole open reading frame of the HIV-1 gp160.

Detailed protocol:

Table I. Amplification primers.

Name	Sequence (5' to 3')
30eu	TATGAAACTTACGGGGATACTTGGG
11ed	CTGCCAATCAGGGAAGTAGCCTTGTGT

The specified restriction sites are underlined in the sequence.

RT-PCR

Add the following to the microcentrifuge tubes placed on ice. Reaction cocktails can be made when multiple reactions are being assembled.

Components	Volume/50 µl	Final Concentration
2X Reaction Mix	25 µl	1x
30eu (10 µM)	1.5 µl	0.3 µM
11ed (10 µM)	1.5 µl	0.3 µM

RT/ Platinum® Taq Mix	1 µl	
Expand[1]	1µl	3.5 U
Autoclaved distilled water	10 µl	
Template RNA	10 µl	30000 copies

[1] Expand High Fidelity Enzyme mix (Roche)

Cycling:

	Temperature	**Time**	**Cycles**
cDNA synthesis	45ºC	45 min	1x
Initial denaturation	94ºC	2 min	1x
Denaturation	94ºC	30 s	15x
Annealing	55ºC	1 min 30s	
Elongation	68ºC	3 min	
Denaturation	94ºC	30 s	20x
Annealing	55ºC	1 min 30s	
Elongation	68ºC	3 min + 20 s cycle elongation for each successive cycle	
Final elongation	68ºC	10 min	1x

Nested-PCR:

PCR product is used as a template for nested PCR amplification with the Expand High Fidelity PCR System (Roche) and the primers 53eu and 52ed containing XbaI and NotI sites respectively (Table II).

The resulting 2.7 kb amplification products include the whole open reading frame of the HIV-1 gp160.

Table II. Amplification primers.

Name	Sequence (5' to 3')
53eu	GCTCTAGAGCTGTGGTCCATAGTAATCATAGAATATAGG
52ed	TACTTTTTGCGGCCGCGCCACCCATCTTATAGC

The specified restriction sites are underlined in the sequence.

Add the following reagents to the microcentrifuge tubes placed on ice. Reaction cocktails can be made when multiple reactions are being assembled.

Components	Volume/50 µl	Final Concentration
Deoxynucleotide mix (10mM)	1 µl	200 µM of each dNTP
53eu (10 µM)	1.5 µl	0.3 µM
52ed (10 µM)	1.5 µl	0.3 µM
Expand High Fidelity buffer, 10x conc. with 15 mM $MgCl_2$	5 µl	1x
Expand High Fidelity Enzyme mix	1µl	3.5 U
Autoclaved distilled water	35 µl	
Template DNA	5 µl	

Cycling:

	Temperature	Time	Cycles
Initial denaturation	94ºC	2 min	1x
Denaturation	94ºC	30 s	15x
Annealing	55ºC	1 min 30s	
Elongation	68ºC	3 min	
Denaturation	94ºC	30 s	20x
Annealing	55ºC	1 min 30s	
Elongation	68ºC	3 min + 20 s cycle elongation for each successive cycle	
Final elongation	68ºC	10 min	1x

Cloning of env coding sequences.

The PCR products are excised from agarose gels, purified, and concentrated with the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). After digestion with XbaI and NotI, PCR amplification products are ligated into the pNL-lacZ/env-Ren vector[1]. The resulting constructs are then used for transfection into XL2-Blue Ultracompetent Cells (Stratagene) and only efficient reactions (>200 clones and <5% religated) are selected. Recombinant plasmids are all purified with the Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA).

Generation of recombinant virus stocks

To generate infectious stocks of recombinant viruses, HEK-293T (AIDS Reagent program, NIH) cells are transfected by calcium-phosphate method using 5µg of recombinant plasmidic DNA carrying different HIV envelopes. The day before

[1] González N, Pérez-Olmeda M, Gacía-Pérez J, et al. Evaluation of HIV-1 tropism using a new and sensitive system based on recombinant viruses. XVII International HIV Drug Resistance Workshop; [abstract 109]. Published in Antiviral Therapy 2008; Supplement 3: P1-P4, A1-178

transfection 5 x 10^5 293T cells are plated in 60-mm diameter Dishes and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (PAN Biotech, Aidenbach, Germany), 2 mM L-glutamine, 100 µg/mL streptomycin and 100 U/ml penicillin. The medium is changed 8h and 24h post-transfection. 48h after transfection supernatants are harvested and clarified by centrifugation at 500 x g for 5 min. Transfection efficiency is monitorised by quantifying luciferase expression in 293T cells (>500.000 RLU/10^5 cells being considered as threshold of good transfection efficiency). Viral supernatants are titrated in U87CD4-CCR5/CXCR4 cells depending on envelope tropism of the recombinant virus. 2x10^4 cells are seeded in triplicate in 96-well plates with different volumes of viral supernatants and luciferase expression in cell lysates is measured 48 hours after transfection. The volume of culture supernatant required to give a luciferase output of 100.000 RLU is selected for neutralisation experiments.

Neutralisation testing

The Mabs and sCD4 were tested at starting concentrations of 25µg/ml and 10µg/ml, respectively, followed by five 2-fold dilutions. Titrated recombinant viruses were pre-incubated with the different concentrations of sCD4 and Mabs fo one hour at 37ºC at a final volume of 300µl. U87CD4-CXCR4 and U87CD4-CCR5 were distributed in 96 well plates (10^4 cells/well) in 100µl volume and recombinant viruses previously neutralised with sCD4 or Mabs were added to cultures (100µl/well). Neutralisation testing for all dilutions are run in triplicate. Luciferase activity in cell lysates was assessed 48 hours after infection using the Luciferase Assay system (Promega, Madison, USA) and a 96-well plate luminometer (Orion, Berthold). IC50, IC75 and IC90 were calculated using the NeutNet calculator. Luciferase activity in cell lysates infected with non-neutralised virus were considered as positive control and luciferase expression in non-infected U87CD4 cells was used as background.