Human Immunodeficiency Virus (HIV) protease region (pol gene) amplification & sequencing.

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This protocol describes the nested RT-PCR approach to protease region (pol gene) amplification and sequencing using integrated proviral DNA or viral RNA as template. This protocol was created to allow for the characterisation of anti-retroviral drug resistance-mutations. The nested strategy allows for better success at sequencing from samples having either low quality, concentration of template or viral load (in the case of RT-PCR). The fragment thus generated partially overlaps on the 3' end with the 5' end of the first reverse transcriptase (RT1) encoding fragment, allowing together with that of the second reverse transcriptase (RTb) and integrase encoding fragments for the assembly of full-pol region contigs.

Oligonucleotides

Name	PCR	Sequence*	bp	%GC	Tm	Position †	Amplicon	Ref.
Prot-FO	1	5´-TAA-TTT-TTT-Agg-gAA-gAT-CTg-gCC-TTC-C-3´	28	39.3	57	2082-2108	652 bp	1
Prot -RO		5'-gCA-AAT-ACT-ggA-gTA-TTg-TAT-ggA-TTT-TCW-gg-3'	32	37.5	58	2703-2734		
Prot -FI	2	5´-TCA-gAg-CAg-ACC-AgA-gCC-AAC-AgC-CCC-3´	27	63	67	2136-2163	514 bp	
Prot -RI		5'-AAT-gCT-TTT-ATT-TTY-TCT-TCT-gTY-AAT-ggC-3'	30	30	56	2621-2650		

***** Reverse oligonucleotide primer sequences given in this table are the reverse-complement of the sequence present in the alignments and as they should be ordered for synthesis.

† Primer binding sites given on table are based on HXB2 reference sequence.

RT-PCR components and conditions

Using Vivantis M-MuLV RT enzyme (Cat: ME2305) and Vivantis Taq DNA Pol (Cat: PL1202).

First strand synthesis (RT)

	cf	1x					
dH_2O	---	2.5 µL					
10 µM Forward oligo	1.125 µM	2.3 µL					
10 µM Reverse oligo	1.125 µM	2.3 µL		RT-1 program in Axygen TC-1			
10 mM dNTPs 10 mM	250 µM	0.5 µL		Total time: 6 min			
RNA	---	10 µL		95 ºC	2 min	1 cycle	
		vf: 17.6 µl	➔	4 ºC	2 min		
↓							
RT Buffer	1x cf	2 µL		RT-2 program in Axygen TC-1			
RT Enzyme 250 IU/µl	5 IU/µL	0.4 µL		Total time: 1:12 hrs			
		vf: 20 µl		38 ºC	60 min	1 cycle	
↓				95 ºC	5 min		
Run RT-2 program in Axygen TC-1			➔	4 ºC	5 min		
↓							

1st Polymerase Chain Reaction (PCR)

	cf	1x					
dH_2O	C*f*	6.02					
10x Buffer PCR	1x	1.25		Run 1st PCR in Axygen cycler			
$MgCl_2$ 50 mM	1.50 mM	0.38		Total time: 1:48 hrs			
dNTPs 10 mM	200 µM	0.25		95 ºC	2 min		
Oligos 10 µM	400 nM	0.50		95 ºC	30 sec	30 cycles	
Taq (Vivantis) 5 UI/µL	0.04 UI/µL	0.10		50 ºC	30 sec		
DNA	-	4.00		72 ºC	1 min		
		vf: 12.5 µl	➔	72 ºC	5 min		
↓							

2nd Polymerase Chain Reaction (PCR)

	cf	1x					
dH_2O	C*f*	9.02					
10x Buffer PCR	1x	1.25		Run 2nd PCR in Axygen cycler			
$MgCl_2$ 50 mM	1.50 mM	0.38		Total time: 2:08 hrs			
dNTPs 10 mM	200 µM	0.25		95 ºC	2 min		
Oligos 10 µM	400 nM	0.50		95 ºC	30 sec	30 cycles	
Taq (Vivantis) 5 UI/µL	0.04 UI/µL	0.10		60 ºC	30 sec		
1st PCR product	-	1.00		72 ºC	1 min		
		vf: 12.5 µl	➔	72 ºC	5 min		

Notes

1. Clean workbench with 0.1% NaOCl 0.1% followed by 70% Ethanol before and after work.
2. Preparation of RT mastermix should only be performed in the RT-PCR room.
3. Preparation of PCR mastermix and addition of sample DNA should only be performed in the pre-PCR enclosure or area.
4. Addition of positive template DNA should be performed on instrument (post-PCR) area.
5. All mastermixes should be prepared on ice to prevent excess evaporation.
6. Vortex and spin all mastermixes before and after aliquoting to PCR tubes.

References

1. Gonzalez R, Masquelier B, Fleury H, Lacroix B, Troesch A, Vernet G, Telles JN. Detection of human immunodeficiency virus type 1 antiretroviral resistance mutations by high-density DNA probe arrays. J Clin Microbiol. 2004 Jul;42(7):2907-12. PubMed PMID: 15243037; PubMed Central PMCID: PMC446276.

Revision history

1.0 Original document.

2.0 Changes to document format only.