Human immunodeficiency virus (HIV) detection & quantitation by qRT-PCR (Taqman).

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This protocol describes the qRT-PCR taqman based approach to HIV RNA detection and quantitation in both two-step (RT and PCR) one-step (RT-PCR) modalities. Qualitative testing (commonly referred to as nucleic acid testing or NAT) is used as a screening test to identify HIV-infected subjects. Quantitation of HIV RNA (viral load measurements) is used as a diagnostic test to evaluate viral set point, determine antiretroviral therapy initiation and response as well as in the clinical assessment of progression to acquired immunodeficiency syndrome (AIDS). The oligonucleotide primers and probe target a conserved *gag* gene-encoding region. The one-step qRT-PCR reaction, while saving time, requires greater amounts of both oligonucleotides and probe.

Oligonucleotides

Oligo	Sequence*	bp	%GC	Tm	Hair	Hm	Ht	Position†	Size	Ref.
HIVrt-F	5'-CTA-gAA-CTT-TRA-ATg-CAT-ggg-TAA-AAg-TA-3'	29	33	55	-1.4	-10	-8.1	1235-63	137 bp	1
Probe	FAM 5'-CAT-TAT-CAg-AAg-gAg-CCA-CC-3' TAMRA	20	50	53				1310-29		
HIVrt-R	5'-gAT-gTC-CCC-CCA-CTg-TgT-TTA-gCA-3'	24	54	61	0.7	-3.3	-8.1	1349-72		

* 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. Hairpin (Hair), homodimer (Hm) and heterodimer (Ht) ΔQ's are given in kcal/mol.

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

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>K03455.1 Human immunodeficiency virus type 1 (HXB2),complete genome; HIV1/HTLV-
III/LAV reference genome

GGGGGAAAGAAAAAATATAAATTAAAACATATAGTATGGGCAAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAG
AAACATCAGAAGGCTGTAGACAAATACTGGGACAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATATAA
TACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAGACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAA
AACAAAAGTAAGAAAAAAGCACAGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGTCAGCCAAAATTACCCTATAGTGCAGA
ACATCCAGGGGCAAATGGTACATCAGGCCATATCACCTAGAACTTTAAATGCATGGGTAAAAGTAGTAGAAGAGAAGGCTTTCAG
CCCAGAAGTGATACCCATGTTTTCAGCATTATCAGAAGGAGCCACCCCACAAGATTTAAACACCATGCTAAACACAGTGGGGGGA
CATCAAGCAGCCATGCAAATGTTAAAAGAGACCATCAATGAGGAAGCTGCAGAATGGGATAGAGTGCATCCAGTGCATGCAGGGC
CTATTGCACCAGGCCAGATGAGAGAACCAAGGGGAAGTGACATAGCAGGAACTACTAGTACCCTTCAGGAACAAATAGGATGGAT
GACAAATAATCCACCTATCCCAGTAGGAGAAATTTATAAAAGATGGATAATCCTGGGATTAAATAAAATAGTAAGAATGTATAGC
CCTACCAGCATTCTGGACATAAGACAAGGACCAAAGGAACCCTTTAGAGACTATGTAGACCGGTTCTATAAAACTCTAAGAGCCG
AGCAAGCT
```

Highlighting indicates forward (green), probe (yellow) and reverse (red) oligonucleotide binding sites.

Two-step (separate RT and qPCR) components and conditions

Dual (separate RT and PCR) uses Vivantis M-MuLV (Cat. No.: ME2305) RT enzyme and TaKaRa Premix Ex Taq Probe qPCR (Cat. No.: RR390L) qPCR Master Mix.

First strand synthesis (RT)

	cf	1x
dH_2O	---	4.85 µL
10 µM oligo mix	1.125 µM	2.25 µL
10 mM dNTPs 10 mM	250 µM	0.5 µL
RNA	---	10 µL
		vf: 17.6 µl

↓

Run RT-1 program in Axygen TC-1 →

Total time: 6 min		
95 ºC	2 min	1 cycle
4 ºC	2 min	

↓

RT Buffer	1x cf	2 µL
RT Enzyme 250 IU/µl	5 IU/µL	0.4 µL
		vf: 20 µl

↓

Run RT-2 program in Axygen TC-1 →

Total time: 1:12 hrs		
38 ºC	60 min	1 cycle
95 ºC	5 min	
4 ºC	5 min	

↓

Quantitative Polymerase Chain Reaction (qPCR)

	cf	1x
TaKaRa ExTaq (probe qPCR)	1x	5
10x PrimeTime HIV (5 uM Primers, 2.5 uM Probe)	150 nM Oligos 75 nM Probe	0.3
ROX Refrence Dye II (50x)	0.5x	0.1
dH20	---	2.6
cDNA	---	2
		vf: 10 µl

↓

Run generic program in Applied Biosystems 7500 →

Total time: 2:10 hrs		
95 ºC	30 sec	
94 ºC	5 sec	40 cycles
60 ºC [3]	34 sec	
RT	∞	

Note 3: Data acquisition

One-step qRT-PCR components and conditions

The One-step qRT-PCR procedure uses TaKaRa One Step Ex Taq qRT-PCR kit (Cat. No.: RR068A).

qRT-PCR		1x
dH2O	-	0.2
2x One Step RT-PCR Buffer II	1x	5
TaKaRa Ex Taq HS (5u/ul)	2 U	0.2
RTase Enzyme Mix II		0.2
10 µM Forward primer	0.6 µM	0.6
10 µM Reverse primer	0.6 µM	0.6
10 µM Probe	0.4 µM	0.4
ROX Reference dye (50x)	0.1x	0.02
RNA		3
		vf: 10 µl

↓

Run Affymetrix program in Applied 7500 →

42 ºC	5 min	
95 ºC	10 sec	
94 ºC	5 sec	40 cycles
60 ºC [3]	34 sec	
RT	∞	

Note 3: Data acquisition

Titration curve preparation for quantitative analysis of viral titres

1. The titration curve and all work with HIV plasmid clone is to be prepared and handled ONLY in the main lab (Post-PCR) area.

2. Unfreeze an aliquot of HIV plasmid which should be at a titre between $2x10^{79}$ and $2x10^{7}$ cp/µL. The vial should clearly be labelled with the original concentration.

3. Pre-label a strip of individual PCR microtubes (cut them out of strips to form individual tubes and caps) with the 1:10 serial dilution titres ($2x10^{6}$ right down to $2x10^{0}$).

4. Add 90 µL of ddH_20 to each of these tubes using a P200 micropipette and filtered tip.

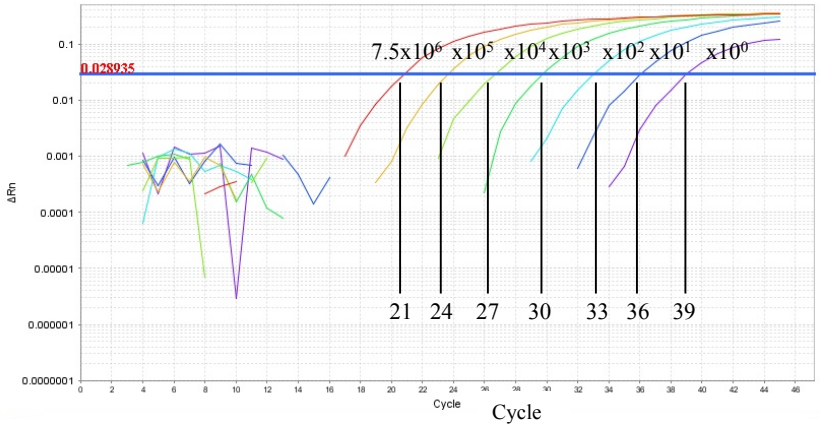
5. Add 10 µL of HIV plasmid (suppose at $x10^{7}$) to the first tube (labelled #6) to prepare the $1x10^{6}$ cp/µl dilution. Mix by pipetting up and down for exactly 20 times without generating bubbles, cap tube and vortex for 10 seconds, spin tube contents down for 10 seconds.

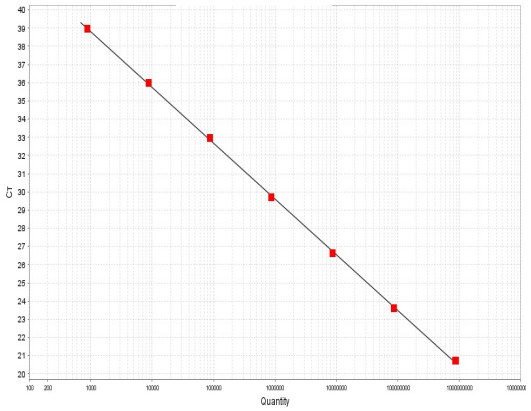
6. Using a new P200 filtered pipette tip, take 10 µL of the previously prepared $x10^{6}$ dilution and add to next PCR tube. Follow the same procedure as in the previous step. Repeat thereon until the last

dilution has been prepared.

7. After preparing the last titre, clean gloves with 70% ethanol and dispose of them in biohazard waste bin. Don new gloves to prepare RT-PCR.

Performance summary





Standard curve: ***m* = - 3.062, Y = 47.98, R^2 = 1**
Limit of detection (LODet): **$7.51x10^0$ cp/µL**
Limit of discrimination (LODis): **$7.51x10^0$ cp/µL**

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 patient 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.
7. In the case of two-step RT-PCR, prepare a 1/10 dilution of the cDNA by adding 180 µL of ddH_20 to the 20 µL RT first strand synthesis reaction of each sample. After capping these tubes, vortex for 10 seconds and spin for 10 seconds before using for the qPCR step.

8. To prepare qPCR reaction, prepare qPCR mastermix in Pre-PCR enclosure within the main lab. aliquot 14 µl of the qPCR mastermix to each of the sample tubes followed by 1 µL of previously generated 1/10 dilution of cDNA.

References

1. Agarwal A, Sankaran S, Vajpayee M, Sreenivas V, Seth P, Dandekar S. Correlation of immune activation with HIV-1 RNA levels assayed by real-time RT-PCR in HIV-1 subtype C infected patients in Northern India. J Clin Virol. 2007 Dec;40(4):301-6. Epub 2007 Oct 24. PubMed PMID: 17962068; PMCID: PMC4230990.

Revision history

1.0 Original document.

2.0 Optimized protocol.

3.0 Changes to document format only.