Standard Operating Procedure for:

WHOLE GENOME AMPLIFICATION OF HIV-1 FROM A SINGLE RNA TEMPLATE

SOP#: CHAVI-MBSC-2

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Revision History:

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1. PURPOSE:

To PCR amplify the whole HIV-1 genome, minus the Long Terminal Repeats (LTRs), from a single viral RNA template (single genome).

2. SCOPE:

This Standard Operating Procedure is applicable to the generation of all whole-genome HIV-1 PCR amplification products, and clones thereof, derived from a single viral template molecule.

3. BACKGROUND AND OVERVIEW OF PROCEDURES:

This protocol is based on SOP # CHAVI-MBSC-1 titled "Single Genome Amplification of HIV-1 Envelope" and CAPRISA SOP titled "RT-Whole Genome PCR" written by Florette K. Treurnicht. This protocol involves the dilution of viral cDNA to a point that, upon setting up multiple replicate PCR reactions with the cDNA as template, the product generated has a high probability of having been amplified from a single genome copy. In this case, according to Poisson's distribution, no more than 30%, or 28 out of a 96-well reaction plate with 2 negative controls, should be positive for amplification product to ensure that 6 of 7 amplified DNAs arise from a single-copy template.

For example, if viral load data is provided, a known amount of viral RNA is purified from plasma using the QIAamp Viral RNA Mini Kit and it is transcribed at a theoretical concentration of 200 copies per μL using a gene-specific primer with Invitrogen's Superscript III—a reverse transcriptase that has been genetically modified to reduce RNase H activity and to maintain cDNA synthesis activity at temperatures (ie. 50-55ºC) required to melt template secondary structure. The viral cDNA is then serially diluted to obtain single copy in a two-step "nested" PCR procedure. Due to variations in sample integrity, stability (ex. freeze/thaw), and serum components, etc., it may be necessary to repeat the PCR reactions at a lower or higher dilution to achieve the desired results.

The resulting positive amplification products are initially subjected to direct DNA sequencing in the highly variable V1-V2 regions to pre-test for sequence homogeneity (lack of multiple superimposed peaks). To confirm single-genome amplification the remainder of the envelope gene is sequenced, followed by the entire amplicon, all of which must be free of any double peaks.

4. ABREVIATIONS AND ACRONYMS

4.1.	PCR	Polymerase Chain Reaction
4.2.	cDNA	complementary DNA
4.3.	RT	reverse transcriptase
4.4.	SSIII	SuperScript III (Invitrogen Co.)
4.5.	DTT	dithiothreitol
4.6.	rxn	reaction
4.7.	PBS	phosphate-buffered saline

5 PRECAUTIONS

5.1 Plasma from acute infection patients can have a viral load in excess of 10 million copies per milliliter! Take precautions as with any infectious agent.

5.1.1 Work with plasma in a sterile containment hood, wear gloves, lab coat and eye protection.

5.1.2 Decontaminate work surfaces and items such as pipettors by wiping with 20% bleach, or 10% Lysol, or 70% Ethanol before and after procedure.

5.2 RNA is extremely susceptible to degradation by RNases.

5.2.1 All tips, tubes, plates and reagents should be of the highest quality and be certified RNase/DNase free.

5.2.2 Aliquot (i.e. 20 μL) and store RNA at –80ºC to avoid repeated freeze/thaw.

5.3 PCR is a very sensitive procedure and can be easily affected by contaminating template.

5.3.1 RNA template preparation, cDNA synthesis and PCR reaction setup should be done in a work area physically separated from any PCR amplified or cloned nucleic acids.

5.3.2 Pipette tips should have a filter barrier.

5.3.3 Transfer of template to nested PCR reactions should be done in a containment cabinet/hood.

6. VIRAL RNA EXTRACTION

NOTE 1: perform in RNA-only clean room.

NOTE 2: this section provides **two options** for Viral RNA Extraction, **A)** using the QIAamp Viral Mini Kit, or **B)** using the Qiagen BioRobot EZ1 Workstation with EZ1 Virus Mini Kit v2.0

6.1 Reagents and Solutions

6.1.1 **Option A)**, QIAamp Viral RNA Mini Kit, (catalogue numbers 52904, 52906, or 52908); **Option B)**, EZ1 Virus Mini Kit v2.0

6.1.2 Ethanol (100%)

6.1.3 Sterile PBS

6.1.4 Patient plasma with viral load quantified

6.2 Materials

6.2.1 Microcentrifuge tubes, 1.5 ml

6.2.2 Sterile RNase-free pipette tips with aerosol barrier

6.2.3 Disposable gloves

6.2.4 Lab coat

6.3 Equipment

6.3.1 Sterile hood

6.3.2 Micropipettors

6.3.3 Microcentrifuge

6.4 **Procedure** **Note: Work with plasma in sterile Biosafety cabinet.**

6.4.1 **OPTION A: QIAamp Viral RNA Mini Kit**

6.4.1.1. Use QIAamp Viral RNA Mini Kit following protocol as written in Handbook with the following adaptations:

6.4.1.2. Normalize the 140 μL plasma sample to contain equivalent viral copies (i.e. 20,000 copies per 140 μL).

6.4.1.2.1. Increase volume to 140 μL with PBS.

6.4.1.2.2. Or, if viral load is too low, concentrate by centrifugation for 1 hr at 23.6K x *g*. Remove supernatant down to 140 μL and resuspend viral pellet.

6.4.1.3. Elute RNA from spin column with 65 μL buffer (stock elution buffer is diluted 1:5 in sterile RNase/DNase free H_2O) to achieve approximately 60 μL net recovery.

6.4.2 OPTION B: Qiagen BioRobot EZ1 Workstation with EZ1 Virus Mini Kit

6.4.2.1. Using pre-prepared carrier RNA stock solution (1.0 ug/μL), prepare working stock by adding 3.6 μL to 56.4 AVE buffer, = 60 μL.

6.4.2.2. In 2 mL sample tubes, normalize the 400 μL plasma sample to contain equivalent viral copies (ie. 20,000 copies per 400 μL)

6.4.2.2.1. Increase volume to 400 μL with PBS

6.4.2.2.2. Or, if viral load is too low, concentrate by centrifugation for 1 hr at 23.6K x g. Remove supernatant down to 400 μL and resuspend pellet.

6.4.2.3. Switch on the BioRobot EZ1.

6.4.2.4. Press START and follow PROTOCOLS menu.

6.4.2.5. Select 400 μL sample volume and 60 μL elution volume.

6.4.2.6. Continue at Step 8 in EZ1 Virus Mini Handbook

7 cDNA SYNTHESIS

7.1 Reagents and Solutions

7.1.1 Superscript III Reverse Transcriptase, Cat. No. 18080-044, Invitrogen Corp. (components: SSIII RT (200 U/μL), 5X First-Strand Buffer, 0.1 M DTT)

7.1.2 Sterile, RNase-free water

7.1.3 10mM dNTP mix

7.1.4 RNA template (Sec. 6)

7.1.5 RNaseOUT, Cat. No. 10777-019, Invitrogen Corp.

7.1.6 20 uM Primer

7.1.6.1 Subtype B: 1.R3.B3R,
5'–ACTACTTGAAGCACTCAAGGCAAGCTTTATTG

7.1.6.2 Subtype C: OFM19, 5'–GCACTCAAGGCAAGCTTTATTGAGGCTTA

7.1.7 RNase H, Cat. No. 18021-014

7.2 Materials

7.2.1 0.5 ml microcentrifuge tubes (RNase-free)

7.2.2 Sterile, RNase-free pipette tips with aerosol barrier

7.2.3 Disposable gloves

7.3 Equipment

7.3.1 Micropipettors

7.3.2 Microcentrifuge

7.3.3 Heat Blocks (shaking), adjustable

7.3.4 Ice Bucket

7.4 Procedure

NOTE 1: Perform in RNA-only clean room.

NOTE 2: Spin down condensate after heat incubation steps.

NOTE 3: Use viral RNA extract equivalent to 5,000 copies.

A Pipette the following components into a 0.5 ml RNase-free tube:

μL/tube		[stock]	[final]
8.50	H2O		
0.50	primer	20 μM	0.25
2.00	dNTP mix	10 mM each	0.5
15.00	RNA template		
26.00	per tube		

B. Place tube in 65ºC heat block for 5', remove to ice for 1'.

C. Add the following components:

μL/tube		[stock]	[final]
8.00	5x Buffer	5x	1x
2.00	DTT	0.1 M	0.005 M
2.00	RNaseOUT	40 u/μL	2 u/μL
1.00	SSIII RT	200 u/μL	5 u/μL
13.00	per tube		
40.00	final volume		

D. Mix and incubate at 50ºC for 1.5 hr.

E. Add 1 μL additional SSIII RT, and then increase to 55ºC for 1.5 hr.

F. Inactivate SSIII RT by heating at 70ºC for 15'.

G. To each tube, add 1 μL RNase H, incubate at 37ºC for 20'.

8 PCR AMPLIFICATION

Note: PCR reaction mixes should be made up and aliquoted in an area free of PCR amplified or plasmid DNA.

8.1 Reagents and Solutions

8.1.1 Sterile H2O, ultrapure

8.1.2 Subtype B Primers:

8.1.2.1 1.U5.B1F: 5'-CCTTGAGTGCTTCAAGTAGTGTGTGCCCGTCTGT

8.1.2.2 1.R3.B3R: 5'-ACTACTTGAAGCACTCAAGGCAAGCTTTATTG

8.1.2.3 2.U5.B4F: 5'-AGTAGTGTGTGCCCGTCTGTTGTGTGACTC

8.1.2.4 2.R3.B6R: 5'-TGAAGCACTCAAGGCAAGCTTTATTGAGGC

8.1.3 Expand Long Template PCR Systems – Roche 11 759 060 001

8.1.4 dNTPs (10mM each).

8.1.5 Diluted cDNA template (above)

8.2 Materials

8.2.1 0.5 ml microcentrifuge tubes

8.2.2 Sterile pipette tips with aerosol barrier

8.2.3 96-well plates

8.2.4 Disposable gloves

8.3 Equipment

8.3.1 Micropipettors

8.3.2 PCR Hood

8.3.3 Thermal cycler

8.4 Establish Appropriate Titration

8.4.1 cDNA Dilutions

8.4.1.1 Perform serial dilutions of cDNA to obtain <30% positivity.

8.4.1.2 Proceed to PCR amplification. If too few/too many positive reactions, repeat PCR with lower/higher dilution.

8.5 First Round Amplification Reaction

8.5.1 Prepare PCR reaction mix for 1-16 reactions (ex. 15 test + 1 negative control).

µL/rxn	x 16		[stock]	[final]
40.00	640	dH2O		
5.00	80	10X buffer with Mg++ (supplied)		
1.75	28	dNTPs	10mM each	0.35 µM
0.75	12	Expand Long Template Enzyme Mix		
0.75	12	1.U5.B1F	20µM	0.3 µM
0.75	12	1.R3.B3R	20µM	0.3 µM
49.00	784			

If using master mix, add 49.00 µL + 1 µL H_2O to the negative well. Add 1.0 µL of 2-fold serially diluted cDNA per well in triplicate wells; for example. 1:2, 1:4, 1:8. 1:16, and 1:32. Once the correct cDNA dilution has been determined, add 15 µL of diluted cDNA to the master mix and then add 50 µL to each of 15 wells.

8.5.2 Place in thermal cycler. Run with following parameters:

	ºC	Time
	94	2 min
10 cycles	94	15 sec
	55	30 sec
	68	8 min
25 cycles	94	15 sec
	55	30 sec
	68	8 min, add 20 sec each cycle
	68	7 min
	4	hold

8.5.3 From all positive wells, save remaining first round in 0.5ml tube at -80ºC for future full-length genome cloning.

8.6 Second Round (Nested) PCR Reaction

8.6.1 Prepare PCR reaction mix for 1-16 reactions (ex. 15 test + 1 negative control).

µL/rxn	x 16		[stock]	[final]
40.00	640	dH2O		
5.00	80	10X buffer with Mg++ (supplied)		
1.75	28	dNTPs	10mM each	0.35 µM
0.75	12	Expand Long Template Enzyme Mix		
0.75	12	2.U5.B4F	20µM	0.3 µM
0.75	12	2.R3.B6R	20µM	0.3 µM
49.00	784			

8.6.2 Pipette 49.0 µL to each well of a 96-well plate. Add 1.0 µL from each of the First Round PCR reaction samples to the corresponding well of the nested PCR plate. Mix by pipetting up and down.

8.6.3 Place in thermal cycler. Run with following parameters:

	ºC	Time
	94	2 min
10 cycles	94	15 sec
	55	30 sec
	68	8 min
25 cycles	94	15 sec
	55	30 sec
	68	8 min, add 20 sec each cycle
	68	7 min
	4	hold

9 Proceed to reaction analysis.

9.1 Agarose Gel Electrophoresis, 3 μL sample. Select all positive wells.

9.2 Sequence DNA directly (0.5 – 1μL per sequencing reaction). Store remaining 2nd round product for future use.

10 Supporting Literature

Note: Printed copies of all below are in the SOP notebook.

10.1 Balfe P, Simmonds P, Ludlam CA, Bishop JO, Leigh Brown AJ. 1990. Concurrent Evolution of Human Immunodeficiency Virus Type 1 in Patients Infected from the Same Source: Rate of Sequence Change and Low Frequency of Inactivating Mutations. Journal Of Virology. 64(12): 6221-6233.

10.2 Edmonson PF, and Mullins JI. 1992. Efficient amplification of HIV half-genomes from tissue DNA. Nucleic Acids Res. 20(18): 4933.

10.3 Jansen R, Ledley FD. 1990. Disruption of phase during PCR amplification and cloning of heterozygous target sequences. Nucleic Acids Res. 18(17): 5153-5156.

10.4 Marton A, Delbecchi L, Bourgaux P. 1991. DNA nicking favors PCR recombination. Nucleic Acids Res. 19(9): 2423-2426.

10.5 Meyerhans A, Vartanian JP, Wain-Hobson S. 1990. DNA Recombination During PCR. Nucleic Acids Res. 18(7): 1687-1691.

10.6 Odelberg SJ, Weiss RB, Hata A, White R. 1995. Template-switching during DNA synthesis by Thermus aquaticus DNA polymerase I. Nucleic Acids Res. 23(11): 2049-2057.

10.7 Paabo S, Irwin DM, Wilson AC. 1990. DNA Damage Promotes Jumping between Templates during Enzymatic Amplification. Journal Of Biological Chemistry. 265: 4718-4721.

10.8 Palmer S, and Kearney M. July 19, 2005. Protocol: Procedures for Single Genome Sequencing (SGS). Personal communication.

10.9 Palmer S, Kearney M, Maldarelli F, Halvas EK, Bixby CJ, Bazmi H, Rock D, Falloon J, Davey RT, Jr., Dewar RL, Metcalf JA, Hammer S, Mellors JW, and Coffin JM. 2005. Multiple, Linked Human Immunodeficiency Virus Type 1 Drug Resistance Mutations in Treatment-Experienced Patients Are Missed by Standard Genotype Analysis. Journal Of Clinical Microbiology. 43(1): 406–413.

10.10 Simmonds P, Balfe P, Ludlam CA, Bishop JO, Leigh Brown AJ. 1990. Analysis of Sequence Diversity in Hypervariable Regions of the External Glycoprotein of Human Immunodeficiency Virus Type 1. Journal Of Virology. 64(12): 5840-5850.

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10.12 Simmonds P, Zhang LQ, Mcomish F, Balfe P, Ludlam CA, Leigh Brown AJ. 1991. Discontinuous Sequence Change of Human Immunodeficiency Virus (HIV) Type 1 env Sequences in Plasma Viral and Lymphocyte Associated Proviral Populations In Vivo: Implications for Models of HIV Pathogenesis. Journal Of Virology. 65(11): 6266-6276.

10.13 Treurnicht, FK. RT-Whole Genome PCR. SOP Nr. n/a. Centre For The AIDS Programme Of Research In South Africa (CAPRISA). Personal communication.