HIV-1 Full Genome Amplification and Sequencing

Francine E McCutchan

Sodsai Tovanabutra

Eric Sanders-Buell

Global Molecular Epidemiology Program
U.S. Military HIV Research Program
Henry M. Jackson Foundation for the Advancement of Military Medicine

Walter Reed Army Institute of Research

Plasma sample preparation and RNA Extraction

Plasma preparation

1. Plasma volume to be extracted will be normalized according to viral load as shown in the table

Viral load	**Plasma volume (ml) to be extracted**
< 10,000	3
10,000 - 30,000	2
30,001 – 70,000	1
70,001- 200,000	0.5
200, 001 – 500,000	0.25

2. Plasma volumes less than 140ul will be extracted without a prior concentration step. For plasma volume greater than 140 µL they will be prepared as follows:

a. Prepare 2ml Sarstedt tubes tubes by marking the 140ul volume level by comparison with a tube filled with water to 140ul. Pipette designated plasma volume into prepared tube.

b. Spin the sample in the high-speed refrigerated microcentrifuge at 20,000 g, 4oC for 1 hr.

c. Carefully remove and discard the supernatant down to 140 µL without disturbing the pellet.

d. Resuspend the pellet by pulse vortexing for 15 sec.

e. If more than 2 ml of plasma is needed, concentrate 1.6 ml of plasma for 1 hr, discard 1.5 ml of supernatant, mix to resuspend the pellet. Add the rest of the plasma (1.4ml) into the tube and spin for further 1 hr. Remove the supernatant down to 140 µL, resuspend the pellet and subject to RNA extraction.

RNA Extraction

Viral RNA is extracted using QIAamp Viral RNA Mini Kit, Cat.# 52904, 52906 or 52908

a. To 140 µL of plasma, add 560 µL buffer AVL containing Carrier RNA. Mix by pulse-vortexing for 15 sec.

b. Incubate at room temperature for 10 min.

c. Briefly spin to remove the drops from the lid and to ensure all contained are at the bottom of the microcentrifuge tube.

d. Add 560 µL of 100% ethanol to the sample, and mix by pulse-vortexing for 15 sec. Briefly spin to remove the drops from the lid.

e. Carefully apply 630 µL the mixture to the QIAamp spin column (in a 2 ml-collection tube) without wetting the rim, close the cap, and spin at 6,000 g for 1 min. Place the QIA spin column in a new clean 2 ml-collection tube.

f. Repeat step e

g. Carefully open the spin column and add 500 µL of Buffer AW1 without wetting the rim. Close the cap, spin at 6,000 g for 1 min. Place the column into a new collection tube.

h. Carefully open the spin column and add 500 µL of Buffer AW2 without wetting the rim. Close the cap, spin at full speed for 3 min.

i. Place the column into a pre-labeled clean 1.5 ml microcentrifuge tube. Carefully open the spin column and add 80 µL buffer AVE. Incubate at room temperature for 5 min, and then spin at 20,000 g for 1 min. Collect the eluent that contains RNA and divide into 2 aliquots of 40 µL.

j. Keep RNA on ice if the RT reaction is to be set up immediately or store at -80oC prior to cDNA synthesis.

cDNA synthesis

Making 4x20 µL of cDNA from each individual is recommended.

a. In a 0.5 mL 0.5 Thermowell tube, prepare the RT master mix 1 without RNA using the volumes calculated in the RT reaction section of the worksheet. Aliquot 5 µL of the master mix in to each reaction tube (12 reaction tubes per a assay batch is recommended; here that will be 4 CNDA reactions per sample x 3 samples). Prepare the RT Master mix 2 without enzymes, mix, and leave the mixture in Cool-Safe or on ice.

b. Transfer tray with the 0.2mL MicroAmp, tubes containing master mix 1 on ice to the template Bio safety cabinet. Add 7µL of RNA to each corresponding reaction tube. Mix and pulse spin briefly to collect samples at the bottom of the reaction tubes.

c. Go to Thermocycler (turn on a Thermocycler and check the cycling conditions at least 30 min prior to use) and select file named cDNA1: 65°C for 5 min and 4°C forever. Place tray in and choose that the volume for reaction is 12µL.

4. During 5 min that the Master mix 1 reaction tubes are in the Thermocycler. Go back to reagent hood and add enzymes ThermoScript and RNaseOUT in to master mix 2 and put in the Cool-Safe or on ice tray in template Bio safety cabinet.

5. Remove the Master mix 1 reaction tubes from Thermocycler at the end of 5 min and immediately place them on ice for at least 2 min.

6. Within 5 minutes, add 8µL of the Master Mix 2 to each tube. Pulse-spin tubes briefly. Place the tray back into Thermocycler and RUN on ST cDNA2.

7. When the RT reaction is done, remove the tubes from Thermocycler and add 1 µL of RNase H to each tube at room temperature. Go to the Thermocycler, select method name ST37: 37°C for 20 min and 4°C forever. Place tray in and choose that the volume for reaction is 21µL. Combine the 4 cDNA reactions from each sample and freeze at -80C or proceed to next step.

Primers:

UNINEF-7' (5'-GCACTCAAGGCAAGCTTTATTGAGGCTT-3': HXB2 number 9605-9632) for 3'- half genome fragment or full genome synthesis.

JL68R (5'-CTTCTTCCTGCCATAGGAGATGCCTAAG-3': HXB2 number 5956 - 5983) for 5'- half genome fragment synthesis.

Reagent kit:

ThermoScript TM Reverse Transcriptase, Invitrogen Corp Cat. # 12236-014, 12236-022

Date:	Name:
PTC225 Thermo-cycler:	Block No:
	File No:

No.	Sample ID	Primers	Master mix 1	1X	X	Cyclings	Regents
1			DEPC H2O	2		**File:CDNA1**	ThermoScr
2			dNTP 10mM	2		65^{0}C for 5 min	Uninef-7'or
4		Uninef-7' or JL68R	primer (50uM)	1			
5			RNA	7			
6							
7			Total	12 ul			
8							
						File: STCDNA 2	
			Master mix 2	**1X**	**X**	60^{0}C for 2 hrs	
						85^{0}C for 5 min	
			5xcDNA buffer	4		10^{0}C forever	
			0.1 M DTT	1			
			Rnase OUT	1			
			DEPC H2O	1.5			
			ThermoScript	0.5			
			Total	8 ul			
						Add 1uL Rnase H **File: ST37** 37^{o}C for 20 min	

cDNA length evaluation by Multiplex PCR

Pool 4x21 µL cDNA into one tube and 1 µL of cDNA is subjected to nested multiplex PCR for gag,

First round primers (20 mM):

Msf12b 5′-AAATCTCTAGCAGTGGCGCCCGAACAG-3′

Bjpol3a 5′-CCAATTATGTTGACAGGTGTAGGTCCTAC-3′

PolJv2 5′-GAAGCYATGCATGGACAAGTRGA-3′

TATU2′ 5′-CTTCTTCCTGCCATRGGARATGCC-3′

JL106 5′-TTCAGCTACCACCGCTTGAGAGACT-3′

Unnef-7′ 5′-GCACTCAAGGCAAGCTTTATTGAGGCTT-3′

1 µL of first round product is subjected to the second round PCR

			Date:	Name:				
			PTC225 Thermocycler:	Block No:			**1st rd**	**Lot N**
				File No:			msf12b	
							bjpol3a	
No	Template ID	ul	Primers		X1	X	polJv2	
1			Msf12b	dH20	7.5		tatU2'	
2			Bjpol3a	dNTP	20		JL106	
4			TATU2’	MgCl	9		dNTP's	
5			JL106	10x PCR Buffer	5		MgCl	
6			Uninef-7’	Amplitaq Gold	1.5		10X Buffer	
7				Total	49		Amplitaq Gold	
							Water	

Second round primers (20 mM):

Gag763 5′-TGACTAGCGGAGGCTAGAAGGAGAGA-3′

Bjpol6 5′-TTACTTTGATAAAACCTCCAATTCCYCCTATC-3′

TATU2′ 5′-CTTCTTCCTGCCATRGGARATGCC-3′

JL105 5′-GTGGAACTTCTGGGACGCAG-3′

TATAnef 5′-GCAGCTGCTTATATGCAGGATCTGAGGG-3′

Reagents: AmpliTaq Gold DNA Polymerase with Gold Buffer and $MgCl_2$ solution, Applied Biosystems Cat# 4311818

			Date:		Name:			
			PTC225 Thermocycler:		Block No: File No:			
8							**2nd rd**	**Lo**
9							gag763	
10					X1	X	BJpol6	
11				dH20	7.5		Polk3	
12			Gag763	dNTP	20		tatU2'	
13			Bjpol6	Primers (20uM)	6		JL105	
14			PolK3	MgCl	9		TATnef	
15			TATU2′	10x PCR Buffer	5		dNTP's	
16			JL105	Amplitaq Gold	1.5		MgCl	
17			TATAnef	Total	49		10X Buffer	
18							Amplitaq Gold	
							Water	

GR GAG1 Cycling conditions

Temperature (°C)	Time	Process
95	10 min	Hot start/Enzyme activation
30 cycles		
95	10 sec	DNA denaturation
60	30 sec	Annealing
72	2 min	Extension
72	10 min	Final extension
4	Forever	Hold

Gel electrophoresis

Visualize PCR products on 1% agarose gel containing ethidium bromide (0.5 µg/ml). The amplicon sizes are as shown in the table.

HIV-1 genome region	Primer Pair (HXB2 position)	Amplicon Approximate Size
Gag	Gag763 (763-788) Bjpol6 (2,534-2,564)	1802 bp
Pol	PolK3 (4,534-4,558) TATU2' (5983-5960)	1450 bp
Nef	JL105 (8,568-8,587) Uninef-7 (9,632-9,605)	1065 bp

cDNA input estimation				
The decision to perform either full-length or half genome amplification depends on the results from the multiplex PCR. The amount of the cDNA input in PCR reaction depends on which bands are present and on the brightness of the bands.				
	Number of bands	**Brightness**	**cDNA Dilution**	**Volume of cDNA in (ml)in PCR reacti**
Full-length amplification				
	3	Strong nef, strong pol and weak gag	neat	2.5, 1, 0.5
	3	Strong nef, strong pol and faint gag	neat	5, 2.5, 1
	2	Strong nef, strong pol	neat	7.5, 5
3' Half-genome amplification				
	2	Strong nef, moderate pol	1 in 3	5, 3, 1
	2	Strong nef, weak pol	1 in 2	5, 3, 1
	1	Strong nef	neat	5, 3, 1
5' Half-genome amplification				
	2	Strong nef, moderate pol	1 in 2	5, 3, 1
	2	Strong nef, weak pol	neat	5, 3, 1
	1	Strong nef	neat	7.5, 5

Long PCR Amplification for Full genome

Full-genome amplification

cDNA is diluted as described before

First round primers:
Msf12b 5′-AAATCTCTAGCAGTGGCGCCCGAACAG-3′
Unnef-7′ 5′-GCACTCAAGGCAAGCTTTATTGAGGCTT-3′

First round reaction:In a reagent preparation Bio safety cabinet, separately prepare the PCR Bottom master mix and Top master mix in a 1.5 mL microcentrfuge tube using the volumes calculated in the workbook (See below). Aliquot 20 µL of the Bottom master mix in to each reaction tube and add two drops of hot DyNAwax using P-1000 on top of it. When the wax is solidified add 20 µL of Top master mix in each reaction tube. Transfer them to a template hood and add cDNA from 1 to 10 µL as calculated (adjust the final volume of cDNA to 10 µL with water).

Second round primers:
Gag763 5′-TGACTAGCGGAGGCTAGAAGGAGAGA-3′
TATAnef 5′-GCAGCTGCTTATATGCAGGATCTGAGGG-3′

Second round reaction:
Bottom and top master mix are prepared as calculated in the worksheet above in a reagent preparation Bio safety cabinet and transferred to the nested PCR Bio safety cabinet. Add 1 µL of the first round PCR reaction mixture. Follow the same direction as stated in the first round when place the reaction tubes in the Thermocycler (File# 13).

Reagents

Expand Long Range dNTPack, Roche Applied Science
Cat#04 829 069 001

Primers as stated.

Date:	Name:	480 Thermocycler	File# : 13

No	Template ID	µL
1		
2		
3		
4		
5		
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		

1st round	Bottom			1st round	Top		
	x1	x			x1	x	Water
water	15.5			water	9.3		dNTPs
dNTPs (10mM)	2.5			5X Buffer w/ MgCl	10		msf12b
msf12b	1			Taq	0.7		uninef-7'
uninef-7	1			total	20		5X buffer w/ MgCl
total	20						Expand Taq

1st round	Lot No:
Water	
dNTPs	
msf12b	
uninef-7'	
5X buffer w/ MgCl	
Expand Taq	

Date:

Name:
480 Thermocycler:
file#:
13

2nd round	Bottom			2nd round	Top		
	x1	x			x1	x	Water
water	15.5			water	18.3		dNTPs
dNTPs (10mM)	2.5			5X Buffer w/ MgCl	10		Gag763
gag763	1			Taq	0.7		TATAnef
TATAnef	1			Total	29		5X buffer w/ MgCl
total	20						Expand Taq

2nd round	Lot No:
Water	
dNTPs	
Gag763	
TATAnef	
5X buffer w/ MgCl	
Expand Taq	

File#13 cycling conditions:

Temperature (°C)	Time	Process
94	2 min	DNA denaturation
10 cycles		
94	10 sec	DNA denaturation
60	30 sec	Annealing
68	8 min	Extension
20 cycles		
94	10 sec	DNA denaturation
55	30 sec	Annealing
68	8 min	Extension
72	10 min	Final extension
4	Forever	Hold

1 µL of the second round PCR reaction mixture is visualized on 0.8% agarose gel eletrophoresis along with DNA ladder. The amplicon size is approximately 8.7 kb.

Long PCR Amplification for 5' Half-genome

5' Half-genome amplification (Fragment 1 or F1)

The method used is as described in the full genome amplification except the primers are different as seen in the worksheet.

cDNA is diluted as described before.

First round primers:
Msf12b 5’-AAATCTCTAGCAGTGGCGCCCGAACAG-3’ Unnef-7’ 5’-GCACTCAAGGCAAGCTTTATTGAGGCTT-3’ Second round primers:
Gag763 5’-TGACTAGCGGAGGCTAGAAGGAGAGA-3’ TatB' 5'-TTCCTGGATGCTTCCAGGGCTCTA-3'

The amplicon size is approximately 5 kb.

Date:

Name:

480 Thermocycler:

file#: 13

No.	Template	uL
1		
2		
3		
4		
5		
6		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		

1st round	Bottom		1st round	Top	
	x1	x		x1	x
water	15.5		water	9.3	
dNTPs (10mM)	2.5		5X Buffer w/ MgCl	10	
Msf12b	1		Taq	0.7	
JL68Rv2	1		total	20	
total	20				

1st round	Lo
Water	
dNTPs	
msf12b	
JL68Rv2	
5X buffer w/ MgCl	
Expand Taq	

Date:

Name:

480 Thermocycler:

file#: 13

2nd round	Bottom		2nd round	Top	
	x1	x		x1	x
water	15.5		water	18.3	
dNTPs (10mM)	2.5		5X Buffer w/ MgCl	10	
Gag763	1		Taq	0.7	
Tat B'	1		Total	29	
total	20				

2nd round	Lo
Water	
dNTPs	
Gag763	
Tat B'	
5X buffer w/ MgCl	
Expand Taq	

Long PCR Amplification for 3' Half-genome

3' Half-genome amplification (Fragment 2 or F2)

The method used is as described in the full genome amplification except the primers are different as seen in the worksheet.

cDNA is diluted as described before.

First round primers:
PolJv2 5’-GAAGCYATGCATGGACAAGTRGA-3’ Unnef-7’ 5’-GCACTCAAGGCAAGCTTTATTGAGGCTT-3’
Second round primers:
PolK3 5’-TAAARYTAGCAGGAAGATGGCCAGT-3’
TATAnef 5’-GCAGCTGCTTATATGCAGGATCTGAGGG-3’

The amplicon size is approximately 5 kb.

Date:

Name:

480 Thermocycler:

file#: 13

No.	Template	uL
1		
2		
3		
4		
5		
6		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		

1st round	Bottom		1st round	Top	
	x1	x		x1	x
water	15.5		water	9.3	
dNTPs (10mM)	2.5		5X Buffer w/ MgCl	10	
PolJv2	1		Taq	0.7	
uninef-7	1		total	20	
total	20				

1st round

Water
dNTPs
PolJv2
uninef-7'
5X buffer w/ MgCl
Expand Taq

Date:

Name:

480 Thermocycler:

file#: 13

2nd round	Bottom		2nd round	Top	
	x1	x		x1	x
water	15.5		water	18.3	
dNTPs (10mM)	2.5		5X Buffer w/ MgCl	10	
PolK3	1		Taq	0.7	
TATAnef	1		Total	29	
total	20				

2nd round

Water
dNTPs
PolK3
TATAnef
5X buffer w/ MgCl
Expand Taq

DNA Multiplication and purification

DNA Multiplication

Four replication of PCR reactions using 1 µL of 1 in 1000 dilution of the second round product and the second round primers are performed to obtain ample DNA amplicon for sequencing.

DNA Purification

1. If there is only one band at the right size (8.7 kb) seen on the agarose gel, the PCR products are purified through Microcon YM-50 columns (Microcon YM-50 columns, Millipore Corporation Cat#42416) as follows:

a. Insert a Microcon sample reservoir into a collect vial. Pipette 50 µL the second round reaction mixture into a sample reservoir without touching the membrane with the pipette tip. Add 150 µL of 1xTE Buffer and close the cap.

b. Spin in Microcentrifuge at 7000g for 3 min. Discard solution in the collect vial.

c. Add 200 µL of 1XTE Buffer into the sample reservoir and spin at 7000g for 3 min. Repeat this step one more time.

d. Separate the sample reservoir from collection vial, place it upside down in a new 1.5 ml microcentrifuge tube and then spin at 14,000g for 3 min to collect concentrate DNA amplicon.

e. 1 µL of the purified DNA is visualized on 0.8% agarose gel electrophoresis and the concentration determined by comparison with a molecular weight DNA markers.

2. If there are other non specific bands apart from 8.7 kb band seen on the agarose gel, the PCR products are gel purified using S.N.A.P. UV free gel purification kit (Invitrogen Catalogue no. K2000-25) on 0.8% agarose gel TAE as follows:

a. Cast 0.8% agarose gel in TAE containing crystal violet at 1.6 ug/ml

b. Add 9 µL of 6X Crystal violet loading dye to 45 µL of PCR product and load onto the gel. Load 10 µL of DNA ladder in loading dye in a separate well to be a marker when the DNA band is not visible.

c. Run the gel at 70 volts for 1 hr in TAE buffer.

e. Place the gel on a white light box. Carefully excise the desired DNA band with a new razor blade and transfer the excised plug to a preweighed sterile 1.5 ml microcentrifuge tube. Use a new razor blade to excise
a new band.

f. Weigh the tube with the gel slice to estimate the volume of the agarose (assume 1 mg is 1ml)

g. Add 2.5 times its volume of Sodium iodide solution and vortex.

h. Incubate at 42^{o}C to melt agarose, mix periodically by vortexing.

i. Add 1.5 volume (band and iodide) of binding buffer and mix well.

j. Assembled a S.N.A.P purification column (A) and collection vial B

k. Load all of the mixture onto S.N.A.P. purification column.

l. Spin at 3000 rpm in a microcentrifuge for 1 min.

m. Pour the contents in the collection vial back to the column and repeat step l.

n. Repeat step m. and discard the liquid in the collection tube.

o. Add 400 µL of 1X Final wash to the S.N.A.P. column and spin at 3000 rpm in a microcentrifuge for 1 min.

p. Repeat step o. and discard the liquid in the collection tube.

q. Further spin at 15000 rpm for 1 min, discard the collection vial and transfer the column to a new collection tube.

r. Add 40 µL of 1xTE buffer, incubate for 1min and centrifuge at 15000 rpm for 1 min. Collect the purified DNA for further analysis.

s. Visualize 1 µL of DNA on 0.8% agarose gel to estimate the amount of purified DNA.

Direct sequencing of the PCR amplification products

Sequencing reactions

Sequencing reaction is performed in a 96 well plate using BigDye Terminator sequencing kits v 3. Sequencing primers used are as listed in the table. Preparation of reaction mixtures and the cycling conditions are as shown in the tables. The sequencing reaction products are purified using purification plates from Centri-Sep 96: Princeton Separation.

The sequencing reaction products are purified using purification plates from Centri-Sep 96 from Princeton Separation as follows:

a. Leave the purification plate at room temperature prior to use. Remove bottom, then top foil from plate. Tape the plate to plastic adapter plate, balance and spin in the Sorvall Table top Centrifuge at 2400 rpm for 2 min to collect buffer. Time begins when centrifuge reaches 500 rpm.

b. Detach purification plate from adapter and discard buffer. Add 96 well plate to adapter, place purification plate on top and tape entire apparatus together. Slowly load sequencing reactions directly over columns in plate without disturbing the gel surface. Direct contact cause matrix cracks and do not contact the sides of the column with the reaction mixture or the sample pipette tip, since these can reduce the efficiency of purification. Spin plate at 2400 rpm for 1.5min.

c. Remove 96 well plate from adapter and dry in spin vacuum for ~30min (high heat). After the plate has dried, add 10μL of Formamide into each well and briefly spin, and load the plate onto the sequencing machine.

Sequence assembly

Sequences obtained from the sequencing machine are edited and cleaned using Sequencher software, version 4.7, Package. Multiple sequences derived from the same amplicon are assembled into a single contig and the homogeneity is reviewed. Multiple superimposed peaks or ambiguous bases are allowed but not more than 1%. All genes of the sequence are examined for open reading frames. The consensus sequence is created and entered into the GDE for further analysis. 5' and 3' half genome sequences derived from the same RNA aliquot are patched with 1.5 kb overlapping. The heterogeneity of the overlapping area is not more than 1%.

Reagents & materials

BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems Cat#4337457

SEQSAVER, Sigma-Aldrich Cat#S3938

Sequencing primers (3.2 pmoles). See sequencing primer lists

Centri-Sep 96: Princeton Separation Cat# CS912

Hi-Di Formamide (deionized), Applied Biosystems Cat#4311320

Sequencing primers 5' to 3'

For full-genome amplicon (48 primers)	
Name	**Sequence 5' to 3'**
Gag763	TGACTAGCGGAGGCTAGAAGGAGAGA
DD	GTATGGGCAAGCAGGGAGCTAGAA
JL19	CTTCTATTACTTTTACCCATGC
GG	AGAAGGCTTTCAGCCCAGAAGT
JL17	CATTCTGCAGCTTCCTCATTGAT
II	ATAATCCACCTATCCCAGTAGGAGAAAT
JJ	CATGCTGTCATCATTTCTTCTA
Q	GTTTCAACTGTGGCAAAGAAGGAC
SP2AS	GGTGGGGCTGTTGGCTCTG
SP1AS	GGATGAATACTGCCATTTGTACTGC
SP7AS	CCCCATATTACTATGCTTTC
SP8AS	CTTGGGCCTTATCTATTCCAT
SP8S	ATGGAATAGATAAGGCCCAAG
BJPOL1	ACAGGAGCAGATGATACAGTA
BJPOL3	GTTGACAGGTGTAGGTCCTAC
AZT2	TTCTGTATGTCATTGACAGTCCAGCT
AZT3	CCAGGAATGGATGGACCAA
AZT6	CAATACATGGATGATTTGTATGTAGG
POL C-	CTAGGTATGGTAAATGCAGTATA
POL G	CCATTTAAAAATCTGAAAACAGG
POL U	ACTTTCTATGTAGATGGGGCAGC
POL K	CCATTTAAAAATCTGAAAACAGG
POL J-	CTACCAGGATAACTTTTCCTTCTA
POL Z	AATTTTCGGGTTTATTACAG
DGPOL4R	CCCATCTACATAGAAAGTCTCTGCT
ACC2	AGGGTCTACTTGTGTGYTATAT
ACC5	TGAAACTTAYGGGGATACTTGG
VIF C	GAYAAAGCCACCTTTGCCTAGTGTT
VIF C-	TTGGTCTTCTGGGGCTTGTTC
TAT B-	TTCCTGGATGCTTCCAGGGCTCTA
GP120-5'	AGAGCAGAAGACAGTGGCAATGA
JL74	CTGTTCTACCATGTTATTTTTCCACATGT
E18	TTGTGGGTCACAGTCTATTATGG

JL95	CACAGTACAATGTACACATGGAAT
JL110	CCAGGGCAAAGAGAAGAGTGGTG
JL104	GGAGGCTTGATAGGTTTAAGAATA
JL89	TCCAGTCCCCCCTTTTCTTTTAAAAA
JL107	GCTTTTCCTACTTCCTGCCAC
ES33	CATTGCCACTGTCTTCTGCTC
793SEQ4	CAGCAGTTGAGTTGATACTACTGG
Z1F	TGGGTCACAGTCTATTATGGGGTACCT
ZAR	GTCCCTCATATCTCCTCCTCCAGGTCT
OFM 54	TTTAATTGTGGAGGGGAATTTTTCT
TU-G	GTCTGGTATAGTGCAACAGCA
TU-J	GGTGAGTATCCCTGCCTAAC
NEF5	GATCTTAGCCACTTTTTAAAAG
NEF6	AGCAGCAGATGGGGTGGGAGCAG
TATAnef	GCAGCTGCTTATATGCAGGATCTGAGGG

Note: Blue primers are forward and red primers are reverse.

For 5' half-genome amplicon (30 primers)	
Name	**Sequence 5' to 3'**
Gag763	TGACTAGCGGAGGCTAGAAGGAGAGA
DD	GTATGGGCAAGCAGGGAGCTAGAA
JL19	CTTCTATTACTTTTACCCATGC
GG	AGAAGGCTTTCAGCCCAGAAGT
JL17	CATTCTGCAGCTTCCTCATTGAT
II	ATAATCCACCTATCCCAGTAGGAGAAAT
JJ	CATGCTGTCATCATTTCTTCTA
Q	GTTTCAACTGTGGCAAAGAAGGAC
SP2AS	GGTGGGGCTGTTGGCTCTG
SP1AS	GGATGAATACTGCCATTTGTACTGC
SP7AS	CCCCATATTACTATGCTTTC
SP8AS	CTTGGGCCTTATCTATTCCAT
SP8S	ATGGAATAGATAAGGCCCAAG
BJPOL1	ACAGGAGCAGATGATACAGTA
BJPOL3	GTTGACAGGTGTAGGTCCTAC
AZT2	TTCTGTATGTCATTGACAGTCCAGCT
AZT3	CCAGGAATGGATGGACCAA
AZT6	CAATACATGGATGATTTGTATGTAGG
POL C-	CTAGGTATGGTAAATGCAGTATA
POL G	CCATTTAAAAATCTGAAAACAGG
POL U	ACTTTCTATGTAGATGGGGCAGC
POL K	CCATTTAAAAATCTGAAAACAGG
POL J-	CTACCAGGATAACTTTTCCTTCTA
POL Z	AATTTTCGGGTTTATTACAG
DGPOL4R	CCCATCTACATAGAAAGTCTCTGCT
ACC2	AGGGTCTACTTGTGTGYTATAT
ACC5	TGAAACTTAYGGGGATACTTGG
VIF C	GAYAAAGCCACCTTTGCCTAGTGTT
VIF C-	TTGGTCTTCTGGGGCTTGTTC
TAT B-	TTCCTGGATGCTTCCAGGGCTCTA

For 3' half-genome amplicon (27 primers)	
Name	**Sequence 5' to 3'**
POL K	CCATTTAAAAATCTGAAAACAGG
POL J-	CTACCAGGATAACTTTTCCTTCTA
POL Z	AATTTTCGGGTTTATTACAG
DGPOL4R	CCCATCTACATAGAAAGTCTCTGCT
ACC2	AGGGTCTACTTGTGTGYTATAT
ACC5	TGAAACTTAYGGGGATACTTGG
VIF C	GAYAAAGCCACCTTTGCCTAGTGTT
VIF C-	TTGGTCTTCTGGGGCTTGTTC
TAT AD-	TTCCCGGRTGKTTCCAGGGCTCTA
GP120-5'	AGAGCAGAAGACAGTGGCAATGA
JL74	CTGTTCTACCATGTTATTTTTCCACATGT
E18	TTGTGGGTCACAGTCTATTATGG
JL95	CACAGTACAATGTACACATGGAAT
JL110	CCAGGGCAAAGAGAAGAGTGGTG
JL104	GGAGGCTTGATAGGTTTAAGAATA
JL89	TCCAGTCCCCCCTTTTCTTTTAAAAA
JL107	GCTTTTCCTACTTCCTGCCAC
ES33	CATTGCCACTGTCTTCTGCTC
793SEQ4	CAGCAGTTGAGTTGATACTACTGG
Z1F	TGGGTCACAGTCTATTATGGGGTACCT
ZAR	GTCCCTCATATCTCCTCCTCCAGGTCT
OFM 54	TTTAATTGTGGAGGGGAATTTTTCT
TU-G	GTCTGGTATAGTGCAACAGCA
TU-J	GGTGAGTATCCCTGCCTAAC
NEF5	GATCTTAGCCACTTTTTAAAAG
NEF6	AGCAGCAGATGGGGTGGGAGCAG
TATAnef	GCAGCTGCTTATATGCAGGATCTGAGGG

File # BEDSEQ

Temperature (°C)	Time	Process
25 cycles		
96	10 sec	DNA denaturation
50	5 sec	Annealing
4	Forever	Hold

	X1	X
Big Dye	4	
Half Dye	4	
DNA Template (90 ng/reaction)	1*	
DeionizeH20	11*	
Primer	1	
Total	21μL (20μL without primer)	

*The number of reactions will depend on number of primers used. Water and template volumes will depend on DNA concentration.