Immunocapture of virion from body fluids

1.0 Purpose: Procedure for immunocapturing HIV virions from blood and seminal plasma, CSF, and cell culture by monoclonal antibody-targeting source cell markers in virion envelopes.

2.0 Equipment

2.1 Roller-mixer: Stuart SRT9

2.2 Microcentrifuge (*e.g.*, Eppendorf 5415D)

2.3 Template Tamer/CleanSpot workstation

2.4 UV cross-linker

2.5 Thermocycler and real-time cycler (*e.g.,* Bio-Rad OPUS)

2.6 Genetic sequence analyzer

3.0 Reagents and Media

3.1 **Monoclonal antibodies:** sourced from Santa Cruz Biotechnology (SCBT.com)

	PRODUCT NAME	CAT. #	ISOTYPE	EPITOPE	APPLICATIONS	SPECIES
1	CD16 (2Q1240)	SC-70548	mouse IgG1	FL (h)	WB,IP,IF,IHC(P),FCM	human
2	CD14 (61D3)	sc-52475	mouse IgG1	Extracellular (h)	IP,IF,IFCM	human
3	PECAM-1/ CD31 (158-2B3)	sc-65260	mouse IgG1	FL (h)	WB,IP,IF,FCM	human
4	CD45RA (4KB5)	sc-20057	mouse IgG1	FL (h)	WB,IP,IF,IHC(P),FCM	human
5	CD45RO (UCH-L1)	sc-1183	mouse IgG2a	FL (h)	WB,IP,IF,IHC(P),FCM	human
6	HLA-DR/DP (HL-38)	sc-51616	mouse IgG2a	FL (h)	WB,IP,FCM	human
7	CD27 (H-260)	sc-20923	rabbit IgG	FL (h)	WB,IP,IF,ELISA	human>mouse, rat
8	CD3-ε (UCH-T1)	sc-1179	mouse IgG1	FL (h)	WB,IP,IF,IHC(P),FCM	human
9	CD2 (MT910)	sc-19638	mouse IgG1	FL (h)	WB,IP,IF,IHC(P),ELISA	human
10	CD21 (A3)	sc-13135	mouse IgG2b	AA 21-260 (h)	WB,IP,IF,IHC(P),ELISA	mouse, human
11	Integrin αX/ CD11c (B6)	sc-46676	mouse IgG1	FL (h)	WB,IP,IF,IHC(P),ELISA	human
12	Iba1 (F-4)	sc-398406	mouse IgG1	FL (h)	WB,IP,IF,IHC(P),ELISA	human
13	CD36 Antibody (SMφ)	sc-7309	mouse IgM κ	Extracellular (h)	WB, IP, IF, IHC(P), FCM	mouse, rat and human
14	CD68 (KP1)	sc-20060	mouse IgG1	Extracellular (h)	WB, IP, IF, IHC(P) and FCM	mouse, rat and human

FL, full length molecule; AA, amino acid region; WB, Western blot; IP, immunoprecipitation; IF, immunofluorescence; IHC, immunohistochemistry; FCM, fluorescence confocal microscopy

3.2 BiotinTag Micro Biotinylation kit (BTAG), Sigma BTAG-1KT
3.3 DMSO, Sigma D5879
3.4 Bicinconinic acid kit, Sigma BCA1-1KT
3.5 µMACS Streptavidin MicroBeads, Miltenyi 120-001-017
3.6 Equilibration Buffer for nucleic acid applications, Miltenyi 120-001-014
3.7 20 µMACS Columns: Miltenyi 120-001-002
3.8 PBS 0.01M pH7.4, CDC #4550
3.9 Tween 20, Bio-Rad, 1706531
3.10 Albumin, Bovine, Sigma A9418
3.11 Wash buffer: PBS +1% BSA + 1% Tween 20
3.12 Blocking buffer: PBS +1% BSA + 1% Tween 20
3.13 Ethanol (96 – 100%)
3.14 DEPC-treated water: Invitrogen REF AM9906
3.15 0.1 M Sodium Phosphate Buffer, pH 7.2, Sigma P9693
3.16 QIAamp Viral RNA Mini Kit: QIAGEN #52906
3.17 QIAquick PCR Purification Kit, QIAGEN #28104 (50 tests)/28106 (250 tests)
3.18 Qubit dsDNA HS Assay Kit, Invitrogen Q32851 (100 tests)/Q32854 (500 tests)
3.19 Elution buffer: 0.01 M Tris-Cl in DEPC-treated water
3.20 BigDye XTerminator Purification Kit, Applied Biosystems #4376486
3.21 SuperScript™ III RT/ Platinum™ *Taq* HiFi: Invitrogen 12574
3.22 RNA Inhibitor, N8080119 Applied Biosystems
3.23 Platinum™ SuperFi II PCR Master Mix : Invitrogen 12368050
3.24 DNase DNA-free kit, Invitrogen AM1906 (for tissue culture supernatants)

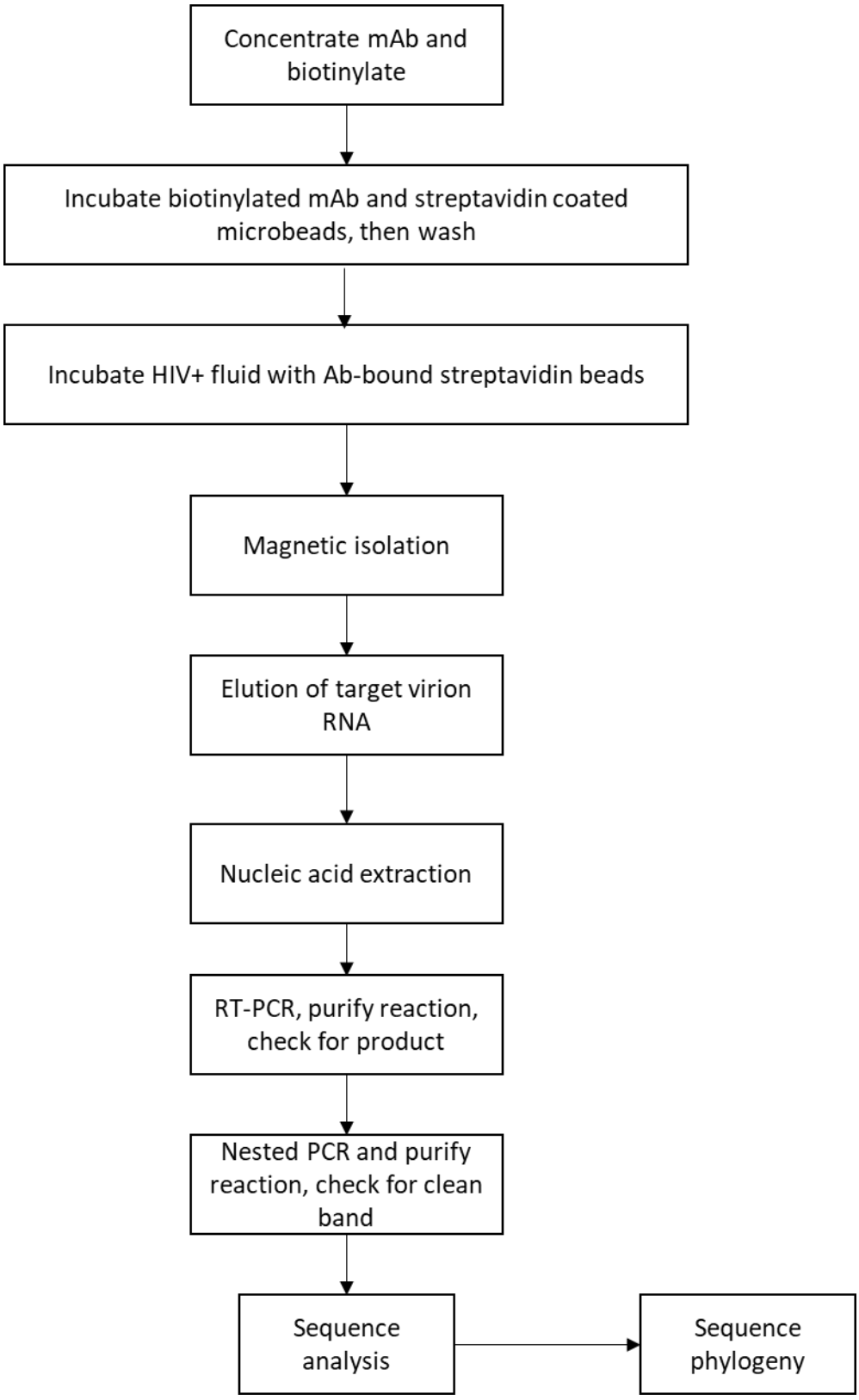
4.0 Supplies, Other Materials

4.1 Amicon Ultra-0.5 filter devices, Millipore UFC5003
4.2 Magnetic Separator: 8 position MACS magnetic stand 007139
4.3 Sterile, RNase-free microcentrifuge tubes, 1.5 mL – 2 mL
4.4 10 µL, 200µL, 1000µL pipette and tips
4.5 RNase-free pipet tips with aerosol barrier
4.6 Immulon II flat well 96-well plates, Nunc #96920
4.7 Microcentrifuge tube racks
4.8 Clear microfilm seals for plates
4.9 96-well hard-shell skirted conical bottom PCR plates
4.10 96-well non-skirted clear conical bottom sequencing plates
4.11 96-well septa mats
4.12 Dedicated spaces for reagent preparation, RNA template, PCR/nested PCR, Real-Time PCR, and sequencing. Gloves must be changed as needed to prevent template contamination.

5.0 Sample Information / Processing (Volume, labeling, handling, storage)

5.1 Fresh, non-frozen biologic sample preferred, stored at 4 ^{o}C and used within 48 hours. If frozen, thaw frozen plasma on ice.
5.2 Aliquot desired input plasma volume from 200 – 400 µL, equivalent to ≤500,000 virus copies, into a 2 mL microcentrifuge tube.
5.2.1 If viral load is unknown, determine copies by qPCR or test on a commercial viral load platform.

6.0 Workflow Chart



7.0 Procedure:

7.1 **Concentrate Antibody**

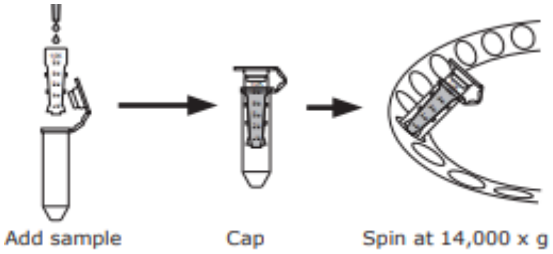
7.1.1 Using Amicon Ultra-0.5 Centrifugal Filter Devices.

7.1.2 Insert the Amicon device into the microcentrifuge tube.

7.1.3 Add up to 500 µL Ab (0.1-0.2 mg/mL) to the filter device and cap it.

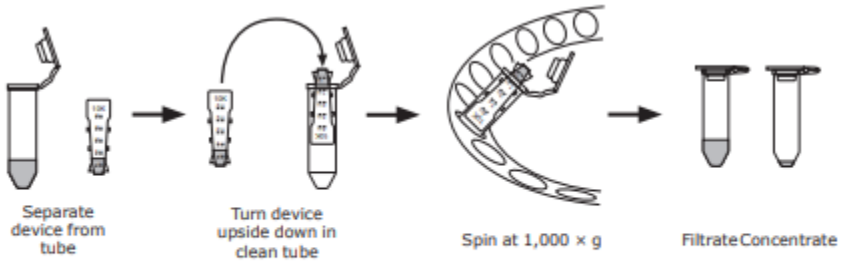
7.1.4 Place capped filter device to the centrifuge rotor, Insert the Amicon Ultra device into the microcentrifuge tube.

7.1.5 Spin the device at 14,000 X *g* for 30 minutes.



7.1.6 Remove the device and place it upside down in a clean tube, place in centrifuge, aligning the open cap strap, toward the center of the rotor.

7.1.7 Spin the device at 1000 X *g* for 2 minutes



7.1.8 Add SPB (0.1 M sodium phosphate buffer, pH 7.2) to reach final volume 100 µL and final concentration: 1~2 mg/1 mL.

7.1.9

7.2 **Antibody Biotinylation (Sigma BTAG)**

7.2.1 Add 30 µL DMSO to the vial of Biotinylation Reagent (BAC-SulfoNHS), and then add 970 µL 0.1M sodium phosphate buffer. The concentration of Biotinylation Reagent is 5 mg/mL.

7.2.2 immediately add 2 µL of Biotinylation Reagent to the antibody solution with gentle stirring.

7.2.3 Incubate with gentle stirring for 30 minutes at room temperature or 2 hours at 2-8 ^{o}C.

7.3 **Isolation of Labeled Antibody (Sigma BTAG)**

7.3.1 Place the column G-50 in a 1.5 ml Eppendorf tube, pre-spin the column for 1 minute at 700 x *g* (3000 rpm).

7.3.2 Add 200 µL PBS (pH 7.4) to the column, spin the column for 1 minute at 700 x g (3000 rpm)

7.3.3 Repeat two times.

7.3.4 Label two of 1.5 ml Eppendorf tube.

7.3.5 Place column in tube 1 and apple the biotinylation reaction mix to the column.

7.3.6 Centrifuge the column for 2 minutes at 700 x *g* and collect flow-through (fraction 1).

7.3.7 Place column in tube 2 and add 200 up to the column, spin the column for 2 minutes at 700 *x g*. collect flow-through (fraction 2).

7.4 **Determine Ab Concentration**

7.4.1 Use Bicinchoninic Acid Kit, 96 well Immulon II plate assay.

7.4.2 Prepare standard curve dilutions:

Protein Ci (µg/mL)	Protein Input Volume (uL)	PBS (µL)	Protein Cf (ug/mL)
1000	-	-	1000
1000	400	100	800
800	375	125	600
600	333	166	400
400	250	250	200
200	250	250	100

7.4.3 Prepare BCA Working Reagent: Mix Reagent A(50) and Reagent B(1)

7.4.4 Add 25 µL protein standard solution, PBS, and Ab samples into well of 96 well plate. Duplicate.

7.4.5 Add 200 µL of BCA working to each well (1:8).

7.4.6 Cover the plate with film and incubate 37 ^{o}C for 30 minutes.

7.4.7 Read the absorbance at 562 nm (540-590 nm).

7.4.8 Calculate mAb concentration against the standard curve.

7.5 **ELISA to Check Biotinylated Antibody**

7.5.1 Using Immulon II 96 well plate

7.5.2 Coat four wells with 100 µL of each biotinylated antibody in PBS (PH 7.4) to the plate, starting 1:100 making 10-fold dilutions. Incubate overnight at 4 ^{o}C.

7.5.3 Wash plate 4 times with PBS+0.05% Tween.

7.5.4 Add 100 µL blocking buffer to each well, incubate at 37 ^{o}C for 1 hour.

7.5.5 Wash plate 4 times with PBS+0.05% Tween.

7.5.6 Add 100 µL of 1:5000 ExtrAvidin_Peroxidase diluted with blocking buffer to each well. Cover plate and incubate at 37 ^{o}C for 1 hour.

7.5.7 Wash plate 4 times.

7.5.8 Add 100 µL TMB substrate to each well.

7.5.9 Develop plate at room temperature in the dark for 15 minutes.

7.5.10 Add 100 µL of stop solution to each well.

7.5.11 Read the absorbance of each well at 450 nm and 550 nm.

7.6 **Streptavidin coated beads_ Biotinylated Ab + HIV→ bead-Ab_HIV complex**

7.6.1 Dilute Biotinylated Ab to 0.4ug/µL with SPB.

7.6.2 Incubate 100 µL of Streptavidin coated beads with 5 µL PBS (negative Ab control) or 2 ug (5 µL of 0.4ug/µL) biotinylated Ab for 10 minutes at room temperature on a roller platform.

7.6.3 Centrifuge bead-Ab complex at 8000 rpm for 10 minutes.

7.6.4 Remove supernatant and wash pellet with 100 µL PBS+1% BSA +1% Tween 20, centrifuge beads Ab complex at 8,000 rpm for 10 minutes. Wash twice.

7.6.5 Add 100 µL Blocking buffer (PBS+1% BSA +1% Tween 20) to the tube and incubate at 4 ºC overnight.

7.6.6 Centrifuge bead-Ab complex at 8,000 rpm for 8 minutes and then remove supernatant.

7.6.7 If working with tissue culture supernatants first DNase treat and inactivate.

7.6.8 Add 200 µL HIV-positive material (plasma, CSF, Semen, Culture or flow-through) to the designated bead-Ab complex and incubate for 30 minutes at room temperature. Mix gently.

7.7 **Prepare µMACs column**

7.7.1 Attach µMACs column to the magnetic multistand.

7.7.2 Add 100 µL equilibration buffer for nucleic acid applications to the column.

7.7.3 Rinse column with 100 µL wash buffer, twice.

7.8 **Binding HIV-bead-Ab complex to the column and collecting the flow-through**

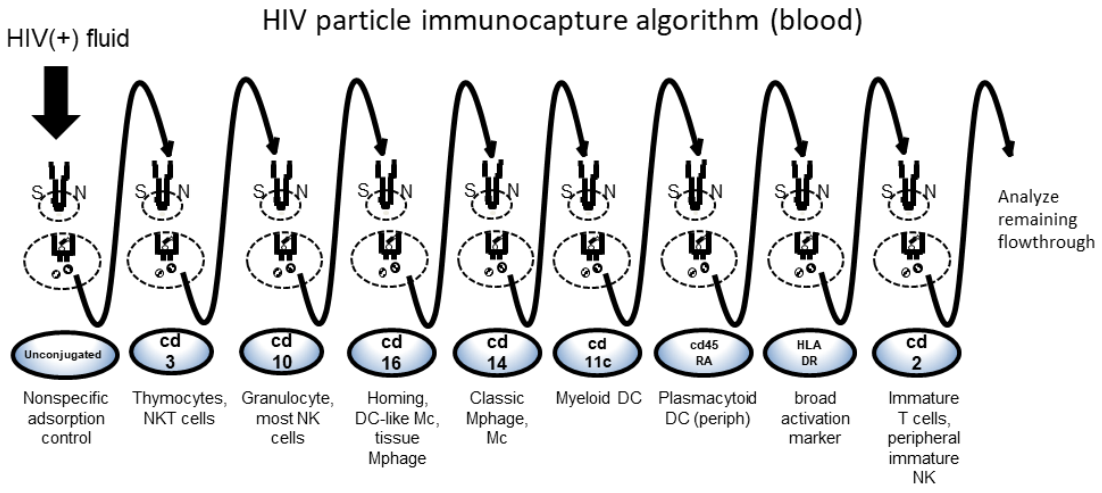
7.8.1 Apply HIV-bead-Ab complex onto the top of column, collecting the flowthrough in a clean microfuge tube or eluting directly into the next tube of biotinylated mAb-bead complex. Let reaction pass through the column completely, captured virus will be retained on the column and flow-through will contain non-target virus (see figure below).

7.8.2 Add 30 µL wash buffer to the column and collect the flow. This accounts for the column void volume and maintain a 200 µL sample volume.

7.8.3 Incubate the flowthrough with next mAb-bead complex for 30 minutes on the roller-mixer at room temperature.

7.8.4 To the just-eluted column, rinse the column 3 times with 400 µL of wash buffer to remove non-specifically bound material, allowing the column drain completely.

7.8.5 Repeat this process until all mAb-bead columns in the series are completed.



7.9 **Elute target virion RNA from the column (using the QIAamp Viral RNA Mini kit)**

7.9.1 After washing the column, place the column of bound virion in a new 1.5 mL Eppendorf tube.

7.9.2 Add 50µL AVL lysis buffer to the column and pass through the column completely.

7.9.3 Add another 150 µL AVL lysis buffer to the column and pass through the column completely.

7.9.4 Add 360 µL AVL lysis buffer to the tube of eluted lysate and incubate tube at room temperature for 10 min. Continue with the extraction kit instructions as follows.

7.10 **RNA extraction: QIAamp Viral RNA Mini Kit**

7.10.1 Add 560 µL ethanol (96–100%) to the sample and mix by pulse-vortexing for 15 seconds. After mixing, briefly centrifuge the tube to remove drops from inside the lid.

7.10.2 Carefully apply 630 µL of the sample solution to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube and discard the tube containing the filtrate.

7.10.3 Repeat this step until all of the lysate has been loaded onto the spin column.

7.10.4 Add 500 µL Buffer AW1. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini column in a clean 2 ml collection tube.

7.10.5 Add 500 µL Buffer AW2. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.

7.10.6 Place the QIAamp Mini column in a new 2 mL collection tube and discard the old collection tube with the filtrate. Centrifuge at 20,000 x *g* (full speed) for 1 min.

7.10.7 Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube. Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µL Buffer AVE equilibrated to room temperature.

7.10.8 Close the cap and incubate at room temperature for 1 minute. Then centrifuge at 6000 x *g* (8000 rpm) for 1 minute.

8.0 RT PCR: SuperScript™ III One-Step RT-PCR System with Platinum™ Taq High Fidelity DNA Polymerase

8.1 Thaw, vortex briefly to mix and centrifuge each component before use.

8.2 Prepare 45 µL reaction mast mix in a PCR workstation.

Component	Volume (uL)
2x Reaction Mix	25
F primer (10 µM)	1
R primer (10 µM)	1
SuperScript™ III RT/Platinum™ Taq High Fidelity Enzyme Mix	2
RNA Inhibitor (40 U/µL)	1
Water	15
Total	45

8.3 Add 5 µL of template RNA. Final reaction volume is 50 µL.

8.4 Gently mix and make sure that all the components are at the bottom of the amplification tube.

8.5 Place the reaction in the preheated thermal cycler programmed as described above. Collect the data and analyze the results.

8.6 Program the thermal cycler to amplify with the following conditions:

1x			40x			1x
25 °C	55 °C	94 °C	94 °C	55 °C	68 °C	68 °C
10 minutes	30 minutes	2 minutes	2 minutes	30 seconds	1 minute	5 minutes

9.0 **Nested PCR (nPCR): Platinum™ SuperFi II PCR Master Mix**

9.1 Thaw, vortex briefly to mix and centrifuge each component before use.

9.2 For each sample, prepare 48 µL reaction master mix in a PCR workstation as follows:

Component	Volume(uL)
Platinum™ SuperFi II PCR Master Mix	25
F primer (10 µM)	1
R primer (10 µM)	1
Water	21
Total	48

9.2.1 Transfer new reaction microfuge tubes and RT-PCR samples to Nested PCR room. Add 2 µL of each RT-PCR sample per tube.

9.2.2 Use a designated 2nd round PCR thermocycler – vortex and quick spin samples before inserting into thermocycler. Amplify with the following conditions (specific for primers used):

1x 98 ºC 2 minutes	30x 98 ºC 15 seconds	56 ºC 30 seconds	68 ºC 1 minute	1x 68 ºC 5 minutes

9.3 DNA is quantified and PCR amplicon size is verified via the Agilent 2200 Tapestation after nested PCR is performed for sequencing. Alternatively, bands can be checked by agarose gel.

9.3.1 Identify samples with clean amplicon bands for further analysis.

10.0 **Perform Sanger sequencing with available platform.**

11.0 **Sequence analysis**

Compare relatedness of HIV sequences in alignment software (*e.g.*, Geneious) and MEGA to generate neighbor-joining trees and perform genetic distance analysis. Perform best model fit (typically Tamura 92 is the best fit)

12.0 **Sample Retention and Storage**

12.1 Frozen plasma specimens should be stored at -80°C until ready for testing.

12.2 Extracted genetic material should be stored at -80°C for long-term storage.

12.3 Amplified RT-PCR can be stored for two weeks at 4°C but should be stored at -80°C for longer storage.

13.0 RT-PCR amplicons should not be stored with clinical samples.

14.0 **Definitions**

Term	Definition
RT	**Reverse Transcription**
PCR	**Polymerase Chain Reaction**
nPCR	**Nested PCR**