Celera ViroSeq™ HIV-1 Genotyping System

1 Principle:

The ViroSeq™ HIV-1 Genotyping System is used for identifying mutations in the pol gene of the human immunodeficiency virus, type one (HIV-1). The entire Protease gene and approximately two thirds of the Reverse Transcriptase (RT) gene in the pol open reading frame are amplified (approximately 1.8 kilobases (kb)). The amplicon is subsequently used as a sequencing template to generate approximately 1.2kb of sequence data. Genotypic analysis of the region of HIV-1 facilitates the study of the relationship between mutations and viral resistance to anti-retroviral drugs, specifically the protease and RT inhibitors. The sequencing data are then used to provide supplemental information regarding the potential HIV-1 susceptibility to current anti-retroviral drugs. Physicians use the data in conjunction with patient clinical data to make treatment decisions. See Hirsch et. al., JAMA v.283 #18 5/10/00 for additional information regarding HIV-1 genotypic analysis.

The genotyping process has five main stages. (1) Isolating the HIV particles from plasma samples followed by the purification of viral RNA. (2) Performing a reverse transcription of the HIV genome using a single primer. (3) Performing PCR amplification of the protease and RT genes from the cDNA made in the reverse transcription reaction. (4) Direct sequencing of the PCR amplification product using seven custom sequencing mixes. (5) Identifying mutations in the protease or RT genes using the ViroSeq™ HIV-1 Genotyping System Software.

The target for reverse transcription is the HIV-1 RNA prepared from plasma. The procedure is carried out using a single primer and the enzyme murine leukemia virus (MuLV) reverse transcriptase. This reaction produces double-stranded RNA-cDNA hybrids which represent the entire HIV-1 pol gene. The cDNA products from the RT reaction are amplified. The resulting amplicon encompasses the entire HIV protease gene, and the 5' end of the RT gene. The PCR procedure employs AmpliTaq Gold® for high specificity and efficiency and AmpErase® UNG anti-contamination chemistry to eliminate false genotyping. The PCR products are sequenced using custom primers formulated with the BigDye Terminator sequencing chemistry. The sequencing products are analyzed on an ABI PRISM® 3100 Genetic Analyzer. DNA base calling is performed by the DNA Sequencing Analysis software. The ViroSeq software imports the sequence data and assembles the six or seven sequences segments into a single sequence, which is then compared to the reference strain.

2 Specimen Requirements:

2.1 Collect a sample by venipuncture (completely expending the vacuum in the tube) into a tube containing EDTA anti-coagulant.

2.2 Check specimen for clots. Clotted samples should be rejected and another sample requested.

2.3 Plasma must be removed from the cells within 2 hours from collection. Store samples at -70^{o} C for up to 6 months until assayed. Avoid repeated freeze/thawing of the sample.

3 Workflow:

3.1 In order to protect unamplified samples from contamination from amplified DNA, different parts of this procedure must be performed in three different, physicall-separated work areas:

3.1.1 Pre-amplification Area 1: This area is used for performing Part 1 – Sample Preparation. A biosafety hood is used to work with infected HIV-1 samples.
3.1.2 Pre-amplification Area 2: This area is used for setting up the reactions for Part 2 – Reverse Transcription and Part 3 – PCR. A hood with a UV light to eliminate DNA cross-contamination is required.
3.1.3 Post-amplification Area 3: This area is used for all steps following PCR amplification.

4 Methods:

4.1 Part 1 – Sample Preparation

4.1.1 Celera's time estimate: for 12 samples, 2.5-3.0 hours, 1.5 hours hands on
4.1.2 Each EDTA human plasma sample is centrifuged to concentrate the virions into a pellet. The supernatant is then removed, the virions lysed, and the viral RNA precipitated with isopropanol and washed with 70% ethanol. Finally, the purified viral RNA is resuspended in RNA diluent.
4.1.3 This step is to be performed in pre-amplification Area 1.

4.1.4 Procedure:

4.1.4.1 Wear the appropriate protective gear: disposable gown, 1-2 pairs of gloves, goggles, and surgical mask. Precool a refrigerated centrifuge and hermetically-sealed rotor to 4ºC.

4.1.4.2 In a 50-mL centrifuge tube prepare 25 mL of 70% ethanol (7.5 mL sterile water and 17.5 mL 100% ethanol). Decant 5 mL (or 1 mL per sample to be extracted) into a 15-mL centrifuge tube, label it "good 70% ethanol", and keep it on ice. Place the rest, labeled "waste", in the hood.

4.1.4.3 Place the frozen plasma samples in a rack in the hood to thaw. Get out one pointed screw-cap 1.5-mL tube for each sample. Assign each sample a number, then label the corresponding tube with that number on the cap, and on one side. On the other side, make an orientation mark for centrifuging.

4.1.4.4 Prepare a low-positive control by combining 50 µL 8E5 postive control with 450 µL negative plasma in a labeled screw-cap 1.5-mL tube with orientation marks for centrifuging.

4.1.4.5 Vortex and pulse spin the thawed plasma samples. Transfer 500 µL of each into the corresponding 1.5-mL tube. Rinse the used pipette tips in the "waste" ethanol before discarding. Spin the samples and control(s) in the pre-cooled centrifuge on for 1 hour at 22,000 x g and 4º C, with the orientation marks out and up.

4.1.4.6 Meanwhile, thaw lysis buffer and RNA diluent from the Sample Preparation Module. If the lysis buffer will not go back into solution, heat it to 37º C in a water bath. Keep the RNA diluent on ice, and the lysis buffer in the hood at room temperature. Pour 600 µL of isopropanol for each sample into a labeled 1.5-mL or 15-mL tube and place in the hood. Get out 4 fine-tipped transfer pipettes for each sample, and place them in the hood (keep them in their packaging in order to keep the tips clean).

4.1.4.7 Immediately after the centrifuge stops, aspirate each sample to dryness with a fine-tipped transfer pipette without disturbing the pellet, which may not be visible. Do this by always keeping the tip of the pipette just below the meniscus, moving it down the wall of the tube opposite the orientation mark. Discard the supernatant in the "waste" ethanol, and rinse the pipettes before discarding. Add 600 µL of lysis buffer to each sample, vortex, and hold at room temperature for 10 minutes. Masks may now be removed.

4.1.4.8 Changing tips after each sample, add 600 µL of isopropanol to each and invert to mix. Spin in a microfuge with a hermetically-sealed rotor at 13,000 x g for 15 minutes, with the orientation marks out and up.

4.1.4.9 Aspirate the samples to dryness with fine-tipped transfer pipettes without disturbing the pellets. Each sample will require 2 transfers.

4.1.4.10 Add 1 mL of the chilled 70% ethanol to each sample, and spin in the microfuge at 13,000 x g for 5 minutes, with the orientation marks out and up. Aspirate again with transfer pipettes. Pulse spin in the microfuge, and aspirate one final time. Allow to air dry with the caps off for 1-5 minutes, until there is no visible moisture.

4.1.4.11 Changing tips after each sample, add 50 µL (100 µL if the viral load is known to be above 15,000 copies/mL) of cold RNA diluent to each, vortex very well, and pulse spin.

4.1.4.12 If not immediately proceeding to the RT reaction, freeze the samples at -70º C for no longer than 2 weeks. Shut down and disinfect the hood.

4.2 **Part 2 – Reverse Transcription**

4.2.1 Celera's time estimate: 2.0 hours, 30 minutes hands-on

4.2.2 A single primer is used for reverse transcription of a 1.8-kb region of HIV-1 *pol*, producing double-stranded RNA-cDNA hybrids.

4.2.3 This step is to be performed in pre-amplification Area 2.

4.2.4 Procedure

4.2.4.1 Get one RNase-free 0.2-µL PCR tube for each sample or control, and place them in the hood. Label a 1.5-mL tube for making RT Mastermix and put it on ice.

4.2.4.2 Thaw RNA samples, including positive and negative controls, vortex for 3-5 seconds, and pulse spin. Hold on ice.

4.2.4.3 Thaw the following reagents out of the RT-PCR Module: HIV RT Mix (blue), RNase Inhibitor (white), MuLV RT (purple), and DTT (yellow). Vortex the RT Mix and DTT for 3-5 seconds, pulse spin all four tubes, and hold on ice.

4.2.4.4 Label the 0.2-µL tubes on the tops and both sides. Transfer 10 µL of each sample into the appropriate tube. Cap the tubes and return the samples

to ice as they are used. In the hood, make enough RT Mastermix for the number of samples being processed plus one extra.

	1 reaction	9 reactions
HIV RT Mix (blue)	8.0 µL	72.0 µL
RNase inhibitor (white)	1.0 µL	9.0 µL
MuLV RT (purple)	1.0 µL	9.0 µL
DTT (yellow)	0.4 µL	3.6 µL
total	10.4 µL	93.6 µL

4.2.4.5 Return RT reagents to ice as they are used. Vortex and pulse spin RT Mastermix. Do not keep RT Mastermix at room temperature for more than 30 minutes. Return the kit reagents to -20º C. Shut down the hood and run the UV lamp to destroy any residual nucleic acids.

4.2.4.6 Take the RT Mastermix and the reaction tubes to the thermal cycler room, and pulse spin the reaction tubes. Place in a 9600 or 9700 thermal cycler on the following program with a reaction volume of 20 µL. Stay with the machine.

65º C	30 seconds
42º C	5 minutes
42º C	60 minutes
99º C	5 minutes
4º C	10 minutes
4º C	∞

4.2.4.7 Pause the thermal cycler immediately after the 42º C for 5 minutes step. The thermal cycler will only hold for 10 minutes, so work quickly to aliquot 10 µL of room-temperature RT Mastermix into each tube. Cap and pulse spin the reaction tubes. Return the tubes to the thermal cycler, and resume the program. Discard the remaining RT Mastermix.

4.2.4.8 Remove the samples promptly when the program finishes. If not proceeding immediately to PCR, turn off the thermal cycler and freeze the samples at -20º C for up to 2 weeks.

4.3 **Part 3 – PCR**

4.3.1 Celera's time estimate: 5.0 hours, 15 minutes hands-on

4.3.2 The cDNA of a 1.8-kb region of HIV-1 *pol*, produced in the RT reaction, is amplified into double-stranded DNA using PCR.

4.3.3 The PCR reaction mix is to be prepared in pre-amplification Area 2.

4.3.4 Procedure

4.3.4.1 Thaw the following reagents out of the current ViroSeq HIV-1 RT-PCR box in the freezer in 838b: HIV PCR Mix (blue), AmpliTaq Gold (gold), AmpErase UNG (green). Vortex 3-5 seconds, pulse spin, and hold on ice.

4.3.4.2 In the hood, make enough PCR Mastermix in a labeled 1.5-mL tube for the number of samples being processed plus one extra.

	1 reaction	9 reactions
HIV PCR Mix (blue)	29.5 µL	265.5 µL
AmpliTaq Gold (gold)	0.5 µL	4.5 µL
AmpErase UNG (green)	1.0 µL	9.0 µL
total	31.0 µL	279.0 µL

Return the reagents to ice as they are used. Vortex and pulse spin PCR Mastermix.

4.3.4.3 Return the kit reagents to the freezer. Shut down the hood and run the UV lamp to destroy any residual nucleic acids.

4.3.4.4 Take the PCR Mastermix to the thermal cycler room and hold there until ready for the PCR reaction. If necessary, bring the frozen RT reaction tubes to the thermal cycler room and thaw.

4.3.4.5 Add 30 µL of PCR Mastermix to each reaction tube. Pulse spin the reaction tubes. Place in the thermal cycler on the following program with a reaction volume of 50 µL.

50º C	10 minutes	
93º C	12 minutes	
93º C	20 seconds	x 40 cycles
64º C	45 seconds	
66º C	3 minutes	
72º C	10 minutes	
4º C	∞	

4.3.4.6 Remove the samples promptly after the program is complete, and turn off the thermal cycler. If proceeding immediately to PCR purification, place the samples on ice; otherwise, freeze the samples at -20º C for up to 2 weeks.

4.4 Part 4a – PCR Purification

4.4.1 Celera's time estimate: 25 minutes

4.4.2 The PCR products are purified in preparation for sequencing. The ultrafiltration membranes in the microconcentrator tubes pass the primers and extra dNTPs, which are discarded, and catch the larger target DNA fragments.

4.4.3 This step is to be performed in post-amplification Area 3.

4.4.4 Procedure

4.4.4.1 If proceeding immediately to DNA quantification, pour the gel now, so that it can set while the PCR products are purified.

4.4.4.2 Place the PCR products (after thawing, if necessary) on ice.

4.4.4.3 For each sample, get out one Microcon Spin Column and two collection tubes. Label the lids of the tubes, and insert the microconcentrators into one set.

4.4.4.4 Pipette 200 µL of sterile water into the top of each microconcentrator, being careful not to touch the membrane.

4.4.4.5 Changing tips after each sample, pipette 100 µL of sterile water into each reaction tube.

4.4.4.6 Pipette the entire 150 µL of each sample into the top of the corresponding microconcentrator, being careful not to touch the membrane. Cap the microconcentrator assemblies.

4.4.4.7 Centrifuge the microconcentrator assemblies for 15 minutes at 500 x g.

4.4.4.8 Pipette 35 µL of sterile water into the top of each microconcentrator, being careful not to touch the membrane. Invert each microconcentrator into the corresponding empty Microcon tube. Don't worry about capping the assemblies.

4.4.4.9 Centrifuge the new microconcentrator assemblies for 5 minutes at 500 x g.

4.4.4.10 Remove and discard the microconcentrators. Discard the tubes of filtrate and cap the tubes of purified PCR products. Either proceed to running the agarose gel or freeze immediately at -20º C for up to 2 weeks.

4.5 Part 4b – Quantifying the DNA

4.5.1 The concentration of DNA in each sample purified PCR products is determined semi-quantitatively by running the samples on an agarose gel alongside a DNA Mass Ladder standard. The DNA Mass Ladder contains known concentrations of different size fragments of DNA. By visually comparing the brightness of each sample's band to that of the mass ladder's bands, the concentration of DNA in each sample is estimated, and a dilution factor to be used for cycle sequencing is chosen.

4.5.2 This step is to be performed in post-amplification Area 3.

4.5.3 Selecting the correct dilution factor is crucial to the success of this assay, as too high a DNA concentration can result in large dye blobs, which will cause the ViroSeq analysis software to reject the sequence data.

4.5.4 Procedure

4.5.4.1 Thaw the DNA Mass Ladder at room temperature for 30 minutes, and vortex for 10-15 seconds.

4.5.4.2 Prepare a 1% agarose mini gel using TBE and containing 0.5 µg/mL of ethidium bromide.

4.5.4.3 Place the gel in a submarine gel cooler and submerse it in 1x TBE buffer containing 0.5 µg/mL of ethidium bromide. Eliminate any bubbles in the buffer around the gel.

4.5.4.4 Load 6 µL of DNA Mass Ladder into one lane of the gel, and 3 µL into the next. For each sample, mix 5 µL of the sample with 5 µL Agarose Gel Loading Buffer, and load into one lane of the gel. Freeze the rest of each sample at -20º C.

4.5.4.5 Electrophorese at 10 V/cm until the bromophenol blue has migrated 5 cm into the gel. Photograph the gel over a UV transluminator.

4.5.4.6 Compare the intensity of the sample bands to that of the known bands of mass ladder in order to choose an appropriate dilution ratio for sequencing:

Brightness of Sample Band	DNA Mass	Dilution
Brighter than lane 1, band 1	>100 ng	1:10
Between lane 1, band 2 and lane 1, band 1	60-100 ng	1:4
Between lane 1, band 3 and lane 1, band 2	40-60 ng	1:2
Between lane 2, band 3 and lane 1, band 3	20-40 ng	Adjust to a final volume of 60 µL

4.6 Part 4c – Cycle Sequencing

4.6.1 The diluted, purified PCR products are sequenced in a thermal cycler. In order to sequence the desired 1.3-kb fragment of DNA with BigDye terminators and the AB 3100, which combination has a 600-base resolution, 7 different primers (4 forward and 3 reverse) are provided, pre-mixed with DNA polymerase, nucleotides, dye terminators, and a magnesium chloride solution. The negative control is not sequenced, as it cannot be run on the sequence detector.

4.6.2 The thermal cycler takes approximately 2.5 hours to run.

4.6.3 Procedure

4.6.3.1 Thaw enough (12 µL for each sample or positive control) of each (A, B, C, D, F, G, H) primer mixture from the Sequencing Module. Place on ice.

4.6.3.2 If necessary, thaw the samples and positive control(s). Dilute each sample or control with good water in the ratio chosen in Part 4b to make around 60 µL of diluted sample in a labeled 1.5-mL tube. Place on ice, and freeze the remaining volumes of the undiluted samples at -20º C.

4.6.3.3 Chart how you are going to organize your 96-well plate. Usually, each sample is assigned to a column, and each primer to a row. If not using a plate, label 0.2-µL PCR tubes with the sample number and primer letter on the cap and both sides, and place the tubes in a rack in the same grid as used for a plate.

4.6.3.4 Pipette 12 µL of each primer mixture into each well (or tube) in that primer's row. Pipette 8 µL of each diluted sample into each well (or tube) in that sample's column. Return the primers and samples to ice.

4.6.3.5 Cap the tubes, or cap the plate with fresh strip caps oriented with the "8" at the bottom of each column (label the top tab of each strip cap with the appropriate column number). Pulse spin, and check that the volumes are consistent. Freeze the primer mixtures and diluted samples at -20º C.

4.6.3.6 Run the plate or the tubes in the thermal cycler on the following program, with a reaction volume of 20 µL.

96º C	10 seconds	x 25 cycles
50º C	5 seconds	
60º C	4 minutes	
4º C	∞	

4.6.3.7 Remove the plate or tubes from the thermal cycler immediately when the program finishes. If not immediately purifying, freeze at in the dark -20º C for up to 3 days.

4.7 Part 4d – Sequence Purification

4.7.1 Using one of three suggested methods, the unincorporated BigDye terminators are removed from the samples so that they do not interfere with sequence detection and analysis. The ethanol/sodium acetate precipitation method is described here.

4.7.2 This step is to be performed in post-amplification Area 3.

4.7.3 Procedure

4.7.3.1 Prepare enough fresh ethanol/sodium acetate solution for the number of wells to be precipitated, plus a few extra.

	1 reaction	10 reactions	64 reactions
100% EtOH	50 µL	500 µL	3.2 mL
3 M NaOAc, pH 4.6	2 µL	20 µL	128 µL
total	52 µL	520 µL	3328 µL

4.7.3.2 Make sure that the strip caps on the plate are labeled with column number and orientation. Remove one strip cap at a time, and pipette 52 µL of the ethanol/sodium acetate solution into each well in that column before removing the next strip cap. Carefully replace the strip caps and seal. Vortex very well.

4.7.3.3 Prepare a balance plate with the correct number of strip caps and approximately 10% less water per well (67 µL per well → 60 µL per well), but do not seal all of the strip caps.

4.7.3.4 Place both that plates onto bench tube racks, to protect the wells from collapsing in the centrifuge. Match the prepared balance plate by mass, then seal the strip caps on the balance plate. Centrifuge at 2000 x g for 20 minutes in a swinging-bucket plate centrifuge. Meanwhile, prepare paper towels and fresh 70% ethanol (approximately 10 mL for 64 wells).

4.7.3.5 As soon as the centrifuge stops, remove the plate, and carefully remove the strip caps without disturbing the pellets. Save the strip caps on a KimWipe. Invert the plate onto two folded paper towels and blot. Place the inverted plate on a fresh folded paper towel (keep the tube rack on the plate). Uncap and blot the balance plate in the same way, and then centrifuge the two plates for 1 minute at 150 x g.

4.7.3.6 A add 150 µL of the 70% ethanol to each well. Seal with the saved strip caps. Add 150 µL of tap water to the same wells in the balance plate, and match the plates (again, sitting inside tube racks) by mass. Seal the strip caps on the balance plate, and centrifuge the two plates at 2000 x g for 5 minutes.

4.7.3.7 As soon as the centrifuge stops, remove the strip caps, invert, blot, and pulse spin as in step 5. Allow the plate to air dry upright in the dark, or for at least 2 hours. If not proceeding immediately to sequence detection, cover the dried plate with clean septa or strip caps and freeze in the dark at -20º C for up to one week.

4.8 Daily Maintenance of the ABI 3100

4.8.1 Turn on the computer attached to the 3100. Log in as 3100user (no password). When the computer has booted completely, power on the sequencer. When the sequencer shows a steady green status light, launch the 3100 Data Collection Software. Wait for the software to boot up.

4.8.2 Prepare 25 mL of fresh sequencing buffer by mixing 2.5 mL of 10x sequencing buffer with 22.5 mL of room-temperature HPLC water in a 50 mL tube.

4.8.3 Put on gloves. Press the tray button on the sequencer. When the tray has finished moving forward, open the instrument doors. Remove the anode buffer reservoir and the capillary tip buffer reservoir.

4.8.4 Discard the used buffer down the drain. Refill the two reservoirs with the fresh buffer just to the fill lines, and carefully replace the septa on the capillary tip buffer reservoir.

4.8.5 Replace the reservoirs on the machine, making sure that the anode buffer reservoir is firmly seated.

4.8.6 Check that the polymer block is free of bubbles, and that all the syringes and ferrules are screwed in tightly. Close the instrument doors.

4.9 Sequence Detection on the ABI 3100

4.9.1 Thaw fresh HiDi formamide and aliquot 10 µL into each well or tube. Cover the plate with septa, or cap the tubes, and return the formamide to the freezer. Vortex samples very well, pulse spin, and set aside to resuspend for at least 30 minutes.

4.9.2 If neither daily maintenance nor weekly maintenance has been done within the last 24 hours, perform daily maintenance on the sequencer.

4.9.3 In the 3100 Data Collection Software, select the “Plate View” tab, then click “New”. Create a new 96-well sequencing (SQ) sheet. Fill in the sheet with the sample names, and the run parameters specified in the “Sequencing Sheet Set-Up” protocol for the dye set you are using. Double-check the sheet, then click “OK”. The new sheet will be loaded into the “Pending Plate Records” frame.

4.9.4 If the samples are in tubes, transfer them to a plate, cover with septa, and pulse spin the plate. Place the plate onto a plate base, and carefully snap on the plate retainer so that all four clips are in place. Check that the plate retainer holes line up with the holes in the septa.

4.9.5 Press the tray button on the 3100. Wait for the tray to finish moving forward, then open the instrument doors and place the plate assembly flat on the tray, with the slanted corner forward. Verify that the appropriate plate position indicator in the 3100 Data Collection Software has turned yellow. Select the appropriate sheet from the “Pending Plate Records” frame, click on the plate position indicator, and wait for it to turn from yellow to green. Close the instrument doors, and wait for the tray to return to its home position.

4.9.6 Switch to the “Status View” tab in the software. If desired, manually fill the capillary array. Press the green “Run” button, and monitor the run until the voltage reaches 12.2 mV and the current reaches the 62-70 µA.

4.9.7 Each sequence detection run (16 samples in two columns) takes approximately 2.5 hours.

Procedure: ACTG Lab Man Celera ViroSeq HIV-1 Genotyping System

Prepared by: ACTG Laboratory Technologist Committee

Preparation Date: 01 June 2004

Date Implemented into the Laboratory: _____

Updated on:

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Reviewed by: Date:

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Supersedes Archived Protocol: DAIDS Virology Manual for HIV Laboratories, Version January 1997