

Bayer HIV-1 TRUGENE Genotyping Method

1.0 Principle

Antiretroviral resistance is an important factor that determines treatment outcome in HIV infected patients. Traditional antiretroviral susceptibility assays and recombinant techniques for phenotypic analysis are time consuming and costly. Genotypic testing can be completed in a few days, is relatively inexpensive and is not limited by *in vitro* culture techniques.

The HIV-1 genotype of an infected patient reflects the presence or absence of mutations in viral RNA associated with antiretroviral resistance. Virology Support Labs may perform this diagnostic assay for the NIH-funded AIDS Clinical Trial Group (ACTG). The ACTG uses this information to qualify the patient for enrollment in clinical trials, to determine appropriate antiretroviral therapy, and to contribute sequence data to the ACTG data analysis group. If the genotype excludes the patient from enrollment in an ACTG study, the physician may use the pattern of resistance to guide the course of therapy.

Bayer's HIV-1 TRUGENE™ Genotyping system is a semi-automated technique used for DNA sequencing and genotyping of HIV-1. Viral RNA extracted from plasma is transcribed to cDNA and amplified in a single-tube RT-PCR reaction. The amplified DNA product is sequenced with a modified Sanger technique, using fluorescent dye-labeled primers and automated laser detection.

Bayer's OpenGene™ UNIX software performs semi-automated data analysis, aligning and interpreting a consensus sequence from the individual forward and reverse primer strands. A 1038 base sequence of HIV-1 DNA is determined, which begins at base 10 of the protease gene and at base 110 of the reverse transcriptase gene. The GeneLibrarian™ Module interprets the HIV-1 genotype using a wild type sequence as a reference. Computer interpretation of the test sequence yields an HIV-1 genotype report, indicating position and specific mutation at recognized mutation sites, as well as synonymous mutations, polymorphisms and unexpected mutations at resistance sites.

The minimum plasma viral load requirement is 1000 copies/mL using standard RNA extraction methods. Research applications using viral concentration techniques have successfully sequenced plasmas with as little as 50 copies/mL of HIV-1 RNA.

This assay is FDA approved for diagnostic purposes.

2.0 Precautions

2.1 Universal Precautions for HIV and Blood Samples

All specimens derived from blood must be handled following the CDC Universal Precautions Guidelines for blood borne pathogens. This includes the use of eye protection, gloves and gowns. Personnel must also be approved to work in the Biosafety Level 2+ or 3 Laboratories before processing samples for the extraction of HIV-1 viral RNA. These policies and procedures are outlined in the BL2+ Laboratory Manual, and the procedure for the extraction of Viral RNA.

2.2 Acrylamide

Sequencing gels contain 6% acrylamide. Supplied in a self-contained syringe-type dispenser, this reagent requires no preparation and minimal handling. Unpolymerized acrylamide is a reactive compound and is considered a neurotoxin. Gloves, eye protection and a lab coat should be worn at all times while handling this material. Unpolymerized acrylamide including "empty" SureFill™ cartridges should be discarded in the satellite waste container and NOT IN THE HOUSE TRASH. All failed cassette loadings should be polymerized in the Gel Toaster™ before discarding in the glass disposal box. Refer to the MSDS and PRB waste disposal policy.

2.3 Formamide Stop Solution

Formamide is contained in the CLIP™ Stop Loading Dye solution. Formamide may carry a risk of infertility or harm to unborn children. Avoid contact with skin or eyes and wear gloves during handling. The small quantities of waste formamide generated by this assay may be discarded in the biohazard waste stream. Refer to the Chemical Hygiene Plan and the Policy for Specimen Collection and Handling.

2.4 High voltage

High voltage is used during the gel runs. Safety switches turn the voltage off whenever the access door to the buffer chambers is opened, but caution should be exercised whenever there is power to the sequencing Towers.

2.5 Laser

The Long-Read Sequencing Towers are classified as a Class-1 laser product as described in the Code of Federal Regulations, 21 CFR Subchapter J; IEC 60825-1:1993 w/ Amendment 1:1997 and EN 60825-1:1994 w/ Amendment 1:1997. Lasers will be inactivated through multiple interlocks when the Tower door is opened. Under no circumstances should personnel attempt to bypass or otherwise defeat the incorporated interlocks.

2.6 UV Light Source

A UV light source in the Gel Toaster™ polymerizes the acrylamide gel in the MicroCel™ cassettes. A safety switch prevents the UV light from being activated unless the cassette tray is closed, but caution should be exercised to protect eyes from direct exposure to UV radiation.

3.0 General Lab Practice for Molecular and PCR Techniques

- 3.1 PCR protocols are highly sensitive to contamination by foreign DNA, RNA and nucleases. Precautions should be taken to keep working area clean. Always wear and frequently change gloves when handling PCR reagents or samples. Work areas and equipment including racks and pipettes should be wiped frequently with 10% bleach and 70% ethanol to minimize cross-contamination.

- 3.2 Certified RNase/ DNA-free plastic vials and tips should be used whenever possible. Barrier tips should be used at all times.
- 3.3 Stock master mixes should be prepared in the PCR prep room. No DNA is allowed in this room. If this area is unavailable, reagents may be prepared in another DNA-free area if thorough cleaning of all work surfaces is performed. Utmost care must be exercised if RT-PCR and CLIP sequencing reagents are handled outside the PCR prep room.

4.0 Equipment and System Overview

4.1 Computer

- 4.1.1 An HP Vectra computer controls the VGI system with monitor and keyboard. The current system includes connections to four (4) Long-Read Sequencing Towers via a Lucent Technologies Port Master 2e local area network hub. E-mail and Internet access is also available through the VGI Vectra computer. A CD-ROM drive is available to upload new versions of the OpenGene™ software.
- 4.1.2 Data files may be downloaded to a 3½" floppy or Zip Disk for backup and archiving.

4.2 PE GeneAmp 9700 Thermal cycler

Perkin Elmer's GeneAmp 9700 thermal cycler, with ramp times of 1° per second is the preferred equipment for RT-PCR and CLIP™ reactions. Other types of equipment may be substituted if the ramp time is equivalent.

4.3 Long-Read Sequencing Towers and Buffer Chambers

Four VGI Long-Read Towers are available and identified as alpha, beta, gamma, or delta machines. These towers are capable of running the short, mid-range or long MicroCel™ cassette plates with the appropriate buffer chambers. The Long-Read Towers are self-contained heated gel electrophoresis units integrated with real time laser detection. The Long-Read Towers are connected to an HP Vectra computer via a Lucent Technologies Port Master 2e local area network hub.

4.4 Gel Toaster™

A Gel Toaster™ is used to fill and then polymerize acrylamide in the MicroCel™ cassettes with UV light. The Toaster has an adjustable cassette holder to accommodate all three available sizes of MicroCel™ cassettes.

4.5 Gel Injector

The Gel Injector attached to the gel plate loading fixture facilitates loading the acrylamide from the SureFill™ cartridge to the glass MicroCel™ cassettes.

4.6 Software and Database

- 4.6.1 The OpenGene™ software program is written with OpenStep, a UNIX application. OpenGene™ contains database management, file management, assay control, and sequence editing modules. OpenGene™ contains the GeneLibrarian™ module for sequence analysis and genotype interpretation.
- 4.6.2 The current version of GeneObjects™ is: GeneObjects™ 3.2 2002/12/17 17:52:48 EST.
- 4.6.3 The current version of GeneLibrarian™ is: HIV.G8 2003/08/06.
- 4.6.4 The current version of Guidelines™ is: DR 3.5 IVD 2003/11/19 Guidelines 8 2003/04/15.

5.0 Reagents and Supplies

- 5.1 Always wear gloves when handling reagents. Never return an aliquot to the original container. Protect reagents from contamination at all times

5.2 General

The following are generic supplies available through various laboratory suppliers. Equivalent supplies may be substituted. Molecular grade reagents should be used and plastics should be sterile and/ or certified RNase/ DNase free.

<u>Item</u>	<u>Source</u>	<u>Item Number</u>
Molecular Grade Water	Ambion	9916
Microvials 2.0 mL	Sarstedt	72.693.105
Microvials 0.5 mL	Sarstedt	72.699.050
PCR tubes 0.2 mL	Perkin-Elmer	N801-0540
Barrier Tips, 10 µL	MBP/Fisher	21-236-3
Barrier Tips, 20 µL	MBP/Fisher	21-236-4
Barrier Tips, 100 µL	MBP/Fisher	21-236-7
Barrier Tips, 200 µL	MBP/Fisher	21-236-1
Barrier Tips, 1000 µL	MBP/Fisher	21-236-28
Barrier Tips, 100 µL I, (Eppendorf)	Fisher	21-381-310B
Barrier Tips, 30 µL I (loading)	Matrix Technologies	7632
Barrier Tips, 10 µL (loading)	Matrix Technologies	7492
PCR 8-well Strips	USA Scientific	1402-2700
TBE Buffer (10x)	BioRad	161-0741
PCR Thermal cycler trays	Perkin-Elmer	40381
PCR Thermal cycler trays	Perkin-Elmer	N801-0541

- *Precision micropipettes, for volume delivery 1-1000 μ L
- *Two Rainin electronic pipettes, Model EDP3 (5-100 μ L) are required. One is dedicated for aliquoting terminator mixes and kept free of any exposure to DNA/RNA. The second is used for aliquoting samples and stop solutions.
- *A Matrix Technologies Impact² 8-well Multi-channel pipette (0.5 to 12.5 μ L) is used for loading samples into the gels.

5.3 VGI Specific Reagents and Materials

The following products are specific to the VGI system and cannot be substituted. Observe storage conditions and expiration dating as defined by the manufacturer.

<u>Item</u>	<u>Source</u>	<u>Item Number</u>
TRUGENE™ Genotyping Kit	Bayer	Box1:05917466,Box2:05918748
MicroCel™ Cassettes (Midsize)	Bayer	06866237
6% SureFill™ Acrylamide	Bayer	06635553

6.0 Standard/Control

- 6.1 All of the specimens processed are known to contain HIV-1, so in that sense there is no normal control as normal human plasma will fail to yield an HIV-1 genotype. Wild-type virus HIV-1_{LAV-1}, without anti-viral resistance mutations, is the “normal control” for this assay. A variety of wild type viruses may be loaded into the GeneLibrarian™ software.
- 6.2 Negative and Positive kit RNA controls supplied with each HIV-1 TRUGENE™ kit should be run with each RT-PCR and CLIP™ run. The manufacturer does not require extraction controls. Control RNA is processed in parallel with the samples from the RT-PCR step through the remainder of the assay. Batch controls must pass before patient genotyping results are reported.
- 6.3 Negative controls must be free of HIV-1 sequence, or the entire run must be repeated. Priming of some areas of the wheat germ RNA in the negative control may result in short areas of sequence in the protease gene.
- 6.4 The kit positive control is a wild type sequence with two polymorphisms in reverse transcriptase: E53E (GAG) and R172K (AAA). Deviation from the expected sequence indicates an error or contamination and must be resolved before accepting the run. If the samples sequenced successfully, the negative control is acceptable and the positive control has no signal, report the results at the discretion of the Director.

7.0 Specimens

- 7.1 Purified HIV-1 RNA is required, 60 μ L preferred.
- 7.2 Refer to the Specimen Collection and Handling Policy and the procedure for the Extraction of Viral RNA.

- 7.3 Maintaining the integrity of sample identity
 - 7.3.1 Upon receipt, plasma specimens are recorded on a master Specimen Log. A Specimen Tracking form is created for each specimen. RNA sample vials are labeled with the patient identification, collection date, extraction date and batch number, and the protocol number,
 - 7.3.2 Sample Processing Log: A sample-processing log should be completed for each RT-PCR/CLIP™ batch with information regarding sample and control identity. An identifier is assigned to maintain sample identity in the RT-PCR tray and CLIP™ tray. Additional processing information specific to each run will be noted here.
 - 7.3.3 Gel Run Log: Each gel run should be recorded with a Gel Run Log, including sample identification, protocol, run and case number. Sequence and batch quality control is documented on this form.
 - 7.3.4 Master Run Log: Upon completion, the patient identification, gel run and case number are recorded on the Master Run Log at the beginning of the gel notebook.
 - 7.3.5 All forms and logs will be filed with the assay records for the protocol.
 - 7.3.6 At each step, verify that the integrity of all sample information is maintained. If clerical errors are discovered, correct and document by initialing. If the integrity of sample identity is compromised at any step, processing must be repeated for the affected steps of the analysis. Document the actions taken. Specimen identity must be documented and verified at every step as part of standard operating procedure.
- 8.0 Procedure: RT-PCR Transcriptions and Amplification
 - 8.1 Reagents should be thawed just before use and returned immediately to -20°C after use. All samples and reagents should be kept on ice after thawing.
 - 8.2 Label a 0.2 mL PCR single PCR tube for each sample and control and place in a rack on ice. Label one 2.0 mL microvial “MMI” and a second 2.0 mL microvial tube “MMII” for the Master Mix solutions and place in a rack on ice.
 - 8.3 RT-PCR thermal cycler program
 - 8.3.1 Verify that the settings for the RT-PCR program are as follows:

RT-PCR Cycle Program			
<i>Reverse Transcription</i>	<i>PCR at two T_m for DNA amplification</i>		<i>Final extension</i>
One cycle of:	20 cycles of:	17 cycles of:	One cycle of:
90°C for 2 min	94°C for 30 sec	94°C for 30 sec	68°C for 7 min
50°C for 60 min	57°C for 30 sec	60°C for 30 sec	4°C Hold forever
94°C for 2 min	68°C for 2 min	68°C for 2.5 min	

- 8.3.2 Select User: vgi
- 8.3.3 Select Method: RTPCR
- 8.3.4 Select volume: 26 µL
- 8.3.5 Select start. Pause the unit when the temperature reached 90°C.

8.4 Prepare Master Mix I (RT-PCR Primers, nucleotides, stabilizers)

8.5 Work in a DNA free area.

- 8.5.1 Vortex to mix reagents gently.
- 8.5.2 Prepare Master Mix I solution in a pre-cooled 2.0 mL microvial according to the following table:

MASTER MIX I (µl)	Number of Samples							
	1	2	3	4	5	6	7	8
RT-PCR Primers	12.0	18.0	24.0	30.0	36.0	42.0	48.0	54.0
dNTP Solutions	3.0	4.5	6.0	7.5	9.0	10.5	12.0	13.5
DTT Solution	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0
RNAse-Inhibitor	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5
	MIX	MIX	MIX	MIX	MIX	MIX	MIX	MIX
Total	18.0	27.0	36.0	45.0	54.0	63.0	72.0	81.0

8.5.3 HIV-1 TRUGENE™ kits contain reagents adequate to test approximately 30 specimens. Record the date and number of samples included in each run on the TRUGENE™ kit box to track the number of assays remaining in the kit. Batches of 6 specimens optimize reagent consumption, but it is not always possible to run a full tray.

8.5.4 Aliquot 9 µL I of Master Mix 1 into a 0.2 mL PCR tube, one tube for each sample and set aside on ice.

8.6 Prepare Master Mix II (Buffer/ RT/DNA Polymerase)

8.6.1 Work in a DNA free area.

8.6.2 Prepare Master Mix II solution in a pre-cooled 2.0 mL microvial according to the following table:

MASTER MIX II (μ l)	Number of Samples							
	1	2	3	4	5	6	7	8
RT-PCR Buffer	20.0	30.0	40.0	50.0	60.0	70.0	80.0	90.0
RNAse-Inhibitor	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5
RT-Enzyme	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0
DNA Polymerase	5.0	7.5	10.0	12.5	15.0	17.5	20.0	22.5
	MIX	MIX	MIX	MIX	MIX	MIX	MIX	MIX
Total	28.0	42.0	56.0	70.0	84.0	98.0	112.0	126.0

8.6.3 Exercise care when rinsing out the pipette tip during the addition of enzymes to the Master Mix II vial because of the viscosity of the enzyme stock solutions. Minimize carry-over on the outside of the tip.

8.6.4 Set Master Mix II aside in ice bucket to be carried to the thermal cyclor. This reagent will be added to samples 7 minutes after RT-PCR reaction begins.

*******All of the following steps should be done outside the PCR prep room*******

8.7 Prepare reaction mixtures for RT-PCR

8.7.1 Thaw RNA samples on ice.

8.7.2 Aliquot 17 μ L I of each RNA sample or control into designated PCR reaction tubes containing 9 μ L I of Master Mix I. Briefly and gently vortex sample and Master Mix I to mix. Exercise extreme care to prevent cross-contamination of samples.

8.7.3 Set the RT-PCR MMI/RNA reaction mix on ice in a PE single vial tray.

8.8 Transfer the reaction mixtures to the thermal cyclor area. Load the PE reaction tray and samples into the 90°C pre-warmed thermal cyclor. Select "Resume" to begin the initial denaturation step.

8.9 Add RT-PCR Master Mix II

8.9.1 Allow the MMI/RNA mix to hold at 50°C for 5 minutes. This initial incubation allows the primers to anneal to the template before the enzymes are added. After 5 minutes at 50°C, pause the thermal cyclor and add 14.0 μ L of Master Mix II to each sample and mix carefully in the pipette 2-3x. Master Mix II should be added one

sample at a time, changing tips between each sample. To minimize the risk of contamination, ensure that gloves are clean, that aerosols are not created, and that only one sample is open at a time. Protect the Master Mix II from contamination.

- 8.9.2 Select "Resume" to reactivate RT-PCR program.
- 8.10 Record run number and start time on Thermal Cycler Run Log. Run time should be about 4 hours.
- 8.11 RT-PCR Run Termination and RT-PCR Product Storage
 - 8.11.1 When the RT-PCR program is complete, the unit will automatically cool to 4°C and hold until manually terminated. Samples can remain unattended in thermal cycler but it is best to transfer samples to refrigerator for storage if not immediately proceeding to the CLIP™ run.
 - 8.11.2 Record end time and run duration on run log and verify run duration ≅ 4 hours.
 - 8.11.3 Check "History" for any errors or problems during run and record in Thermal Cycler Run Log.
 - 8.11.4 Label the tray and store the RT-PCR product at -20°C or transfer to ice and proceed to CLIP™ reaction.
- 9.0 Procedure: CLIP™ Sequencing

9.1 Thaw reagents just before use. Return immediately to -20°C storage after use. All samples and reagents should be held on ice during set up. Assemble sequencing tubes and processing tubes on ice to pre-cool. Label all tubes before starting.

9.2 Verify that the settings for the CLIP™ Sequencing program are as follows:

<i>CLIP™ Cycle Program</i>		
<i>Denaturation</i>	<i>Cycle-Sequencing</i>	<i>Final Extension</i>
Hold:	30 cycles of:	One cycle of:
94°C for 5 min	94°C for 20 sec	70°C for 5 min
	56°C for 20 sec	4°C Hold forever
	70°C for 1.5 min	

- 9.2.1 Select User: vgi
- 9.2.2 Select Method: clip
- 9.2.3 Select Volume: 12µL

9.2.4 Select "Start". Allow the unit to reach 94°C, then select "Pause" to hold at this temperature until ready to load.

9.3 Aliquot Terminator Mixes

9.3.1 Work in a DNA free area.

9.3.2 Vortex reagents to mix gently.

9.3.3 Sixteen di-deoxy terminator mixes are required for each sample: four ddNTP/dNTP mixes for each of four primer pairs (Protease, RT-beginning, RT-middle, and Protease 2). Each primer pair consists of sense and anti-sense primers labeled with Cy™ 5 and Cy™ 5.5 dyes. A maximum of 6 tests or 4 unknowns and two controls can be run per CLIP™ plate.

9.3.4 Load two 8-tube strips per sample into the PE PCR tray/ retainer assembly and place on ice. Cover every other row with lids to prevent contamination and loading errors. An additional clean plastic cover may be used to prevent contamination of open wells during processing.

9.3.5 Place the tray with 8-tube strips in horizontal position and the corner notch at lower right. Refer to CLIP™ sequencing template diagram for the loading sequence of terminator mixes. The loading sequence must match the assay design sequence in the VGI Run Setup software.

9.3.6 Use the DNA-free Eppendorf electronic pipette to load 7µL of each terminator mix into the 8-tube strips according to the template. Load terminator mixes directly to bottom of each well to minimize cross contamination in subsequent loading steps. Change tips between each terminator mix.

9.3.7 After loading all 16 terminator mixes, cover wells and set aside on ice.

9.4 Prepare CLIP™ Master Mix

9.4.1 Work in a DNA free area.

9.4.2 Prepare the CLIP™ Master Mix in a 2.0 mL microvial according to the number of samples being processed:

	Number of Samples							
CLIP™ MASTER MIX (µl)	1	2	3	4	5	6	7	8
Molecular grade H ₂ O	108.9	181.5	254.2	326.8	399.4	472.0	544.6	631.8

CLIP™ Buffer	27.5	45.9	64.2	82.6	100.9	119.3	137.7	159.6
AmpliTaqFS-PP	5.2	8.6	12.1	15.6	19.0	22.5	25.9	29.9
	MIX	MIX	MIX	MIX	MIX	MIX	MIX	MIX
Total	141.6	236.0	330.5	425.0	519.3	613.8	708.2	821.3
Remainder	46.2	45.2	44.3	43.4	42.3	41.4	40.4	58.1

9.4.3 Aliquot 95 µL of the CLIP™ Master Mix into a labeled, pre-cooled 0.5 mL microvial for each sample.

*******All following steps should be done outside the PCR Prep Room*******

9.5 Add samples for CLIP™ Sequencing run

9.5.1 Add 5µL I of sample RT-PCR product to the corresponding 1.5 mL microvial containing the CLIP™ Master Mix. Cap tightly and vortex gently for 1-2 seconds.

9.5.2 Use the Eppendorf electronic pipette to dispense 5 µL of sample/CLIP™ Master Mix to each of the designated tubes of terminator mixes. Dispense the sample/CLIP™ mixture on the side of the tube, to ensure delivery of the entire volume and to prevent cross-contamination.

9.5.3 Repeat for each sample and control.

9.5.4 Cap the tubes and seal.

9.5.5 Tap the PCR tray until the contents fall to the bottom of the tubes. Gently mix by vortexing. Tap the tray to settle contents at the bottom of the tubes.

9.5.6 Place PCR tray on ice and transfer to the thermal cycler area.

9.6 Load the tray into the pre-heated thermal cycler. Select “Resume” to reactivate the cycle.

9.7 Record run number and start time on Thermal Cycler Run Log.

9.8 After approximately 2 hours the CLIP™ sequencing run will terminate and hold at 4°C.

9.8.1 Record end time and duration of run on the Thermal Cycler Run Log.

9.8.2 Transfer CLIP™ products to ice.

9.9 Termination of the CLIP™ Sequencing Reaction

9.9.1 The activity of the CLIP™ reaction enzymes must be terminated with Stop Loading Dye. This multipurpose solution serves as a

reaction stop agent, DNA denaturing agent and as the loading dye for the sequencing gels. Caution: contains formamide.

- 9.9.2 Use the Eppendorf electronic pipette to dispense 14 μ L Stop Loading Dye per well. Use caution when uncapping the tubes to minimize aerosol production. Keep caps in order to prevent cross-contamination.
- 9.9.3 Recap tubes and gently tap to settle the contents. Gently vortex to mix. Tap to settle contents. Place on ice and transfer to thermal cycler area.
- 9.9.4 Select "Denature" method from the thermal cycler Vgi user menu: 3 minutes at 85°C, followed by a hold at 4°C.
- 9.9.5 Transfer to ice and proceed to electrophoresis and detection or store at 4°C. Protect from light.

10.0 Procedure: Electrophoresis and Detection

10.1 Preparation of Tris-Borate-EDTA Running Buffer

- 10.1.1 1x TBE: 0.089M Tris, 0.089 M Borate, 0.002M EDTA. Any commercial source of TBE buffer resulting in an equivalent composition is acceptable.
- 10.1.2 Preparation of 1x TBE from 10x powder stock, USB #70454:
 - 10.1.2.1 Dissolve the pre-weighed powder stock in 200 mL of ddH₂O. Record date made, discard date and initials when made. 10x stock can be stored at room temperature for 1 month.
 - 10.1.2.2 Prepare 200 mL of 10x TBE. Add 1800 mL of ddH₂O, for a final volume of 2000 mL. This buffer solution is ready to use for gel runs. Record date made, discard date and initials when made. Solutions should be discarded after 1 month.

10.2 Electrophoresis and Detection

- 10.2.1 In the OpenGene™ program, at the "Open Existing File" prompt, select "Cancel."
- 10.2.2 Turn on the Long-Read Towers to begin heating. NOTE: Towers should be turned on after the computer or a communications error message will result.
- 10.2.3 Mount the buffer chambers in the Towers.

- 10.2.4 Allow SureFill™ acrylamide cartridge(s) to equilibrate to room temperature for at least 30 minutes. Keep in an upright position.
- 10.2.5 From OpenGene™ menu options select “Tools/ Run Setup Wizard.”
 - 10.2.5.1 Select “Setup”: P2 Performance. This selects the loading template to match the terminator mix loading template.
 - 10.2.5.2 Select “Database”: ACTG
 - 10.2.5.3 Select “Sequencer”: Long-Read Towers
 - 10.2.5.4 Fill in Month (mmyyyy)
 - 10.2.5.5 Fill in Patient ID
 - 10.2.5.6 Fill in Sample ID
 - 10.2.5.7 Fill in Date Drawn (mmdyyy)
 - 10.2.5.8 Select appropriate “Protocol” for the sample from the pull-down menu.
 - 10.2.5.9 Select “Create New Run”.
 - 10.2.5.10 Record the assigned Run number on the Run Log Sheet. Fill in the Run Log with sample and run information.
 - 10.2.5.11 Select a Long-Read Tower (alpha, beta, gamma, delta) and click “Connect.”
 - 10.2.5.12 Select “Sequencer Control” tab. Verify status “Connected to...(tower).” Ensure that no error messages appear in the message box. An error message will occur if the selected tower is in use for another run, if the tower is off, or if it is not connected to the network. “Network” should be highlighted from the pull-down menu. Resolve any errors before continuing.
 - 10.2.5.13 Select “Assay Information” tab, select a primer group and verify assay information. Return to “Sequencer Control” Tab.
 - 10.2.5.14 Ensure run parameters are as follows:

Temperature: 60°C
Voltage: 2000

Laser Power: 50%
Sample interval: 0.5 seconds
Run time: 50 minutes
Peak Distance: 10

- 10.2.5.15 Select "GO" from Sequencer Control window.
- 10.2.5.16 At the "ALERT" prompt select "Prerun."
- 10.2.6 A yellow Prerun indicator light on the front panel of Tower and the message box on the screen will indicate the Prerun has started. After about 15 minutes, illumination of a Green light will indicate the Tower has reached running temperature. The message box prompts "Load Time" when both indicator lights (green and yellow) become illuminated. Gels must not be loaded until this has occurred. Occasionally an "ALERT- NO CURRENT" prompt will appear on screen when Tower temperature reaches 60°C. Click "Continue" as often as required, and the message will disappear. DO NOT click "IGNORE." This will cause Tower to ignore any problems that may arise during gel run.
- 10.2.7 Minimize the window and repeat Run Setup Wizard for next samples.
- 10.3 Cast Acrylamide Gels
 - 10.3.1 While the Long-Read Towers are warming up, assemble MicroCel™ cassettes with combs and clips.
 - 10.3.2 Ensure that the MicroCel™ cassettes are clean in the laser detection area. Handle MicroCel™ cassettes on the long edges, avoiding contact with the open ends of the cassette. Smudges, lint, and bubbles in the detection area may interfere with sequence detection.
 - 10.3.3 Tap the SureFill™ cartridge a few times to allow air bubbles to move to the top of the cartridge. Attach the Gel Injector and discharge a small amount of acrylamide to release bubbles. Discard unpolymerized gel in the satellite waste container.
 - 10.3.4 Attach the SureFill™ cartridge to the gel casting assembly with the tubing supplied with the cartridge.
 - 10.3.5 Discharge a few drops of acrylamide through the assembly into a Kimwipe to remove bubbles and check connection for leaks. Discard unpolymerized gel in the satellite waste container. Check the gel loading assembly gaskets for acrylamide debris and crystallized urea. Clean as needed.

- 10.3.6 When the Towers are ready to load, insert a clean, assembled MicroCel™ cassette into the gel casting assembly with the top (comb) of cassette to the left and bottom of cassette to the right at the SureFill™ cartridge connection.
- 10.3.7 Align the cassette and secure by tightening the locking wheel. To prevent acrylamide spillover, place a piece of transparent tape may be placed across top of MicroCel™ cassette just under the wells. Place protective Lucite splash cover over bottom portion of gel casting assembly.
- 10.3.8 With a quick, steady squeeze on the Gel Injector trigger, fill cassette just until acrylamide spills from the wells.
- 10.3.9 Ensure that the cassette is completely filled and free of excess bubbles.
- 10.3.10 Reject any cassettes with excessive or large bubbles.
- 10.3.11 Polymerize acrylamide before discarding rejected cassettes and discard in glass disposal box.
- 10.3.12 Press the “Start” button on the front panel of the Gel Toaster™ to activate UV lights, and begin polymerization of the acrylamide gel. A beeper will sound and UV lights will automatically shut off after 4 minutes when polymerization is complete. Start the Prerun within 5 minutes to maintain gel quality.
- 10.3.13 Pull tray out to stop the beeper and reset the timer.
- 10.3.14 Remove the filled cassette. Gently remove tape and discard. Remove the comb and clips and place in water to rinse. Carefully blot excess gel from the cassette with a Kimwipe.
- 10.4 Prepare the polymerized MicroCel™ cassette for sample loading: “Prerun”
 - 10.4.1 Insert the MicroCel™ cassette into the Tower and check alignment of the lanes with the laser detectors. Lane separators on MicroCel™ cassette should fall midway between detectors. Lock in place.
 - 10.4.2 Fill both upper and lower buffer reservoirs to fill line with 1x TBE buffer, approximately 150 mL.
 - 10.4.3 To prevent current arcing in the buffer chamber, avoid spills and over-filling. Cover the upper buffer chamber with the chamber cap to prevent evaporation and condensation in the Tower.
 - 10.4.4 Close the Tower door to activate voltage and laser power.

- 10.4.5 Verify settings on screen. Current should read 8 to 9 mA.
- 10.4.6 Set timer for 3-5 minutes for gel "Prerun." To maintain gel quality, do not Prerun gel for more than 10 minutes. Continue to prepare gels as needed.
- 10.5 Load the gel
 - 10.5.1 After 3-5 minutes of Prerun, open the Tower and disperse any bubbles at the loading wells.
 - 10.5.2 Load wells 1-8 with 1-2 μ L sample. Close the Tower door. The samples will begin to move into the gel. Load the remaining wells 9-16 with 1-2 μ L sample. Cover the upper buffer chamber with the chamber cap. Close the Tower door and push the "Run" button under the green indicator light bearing the "diamond" symbol. This should extinguish the yellow "Prerun" light. The message display on the Tower will reset to "Runtime 00:00", and the on-screen message box will indicate, "Run Started Correctly." Do not open the Tower door until the run is complete.
- 10.6 Initial run evaluation
 - 10.6.1 Select "Raw Data Viewer."
 - 10.6.2 Examine "Raw Data Viewer" for background signal levels. Ideally, background should be below 50 units. Backgrounds higher than 10,000 units indicate excessive light scattering at the detector. The signal for this lane may be compromised.
 - 10.6.2.1 Generally this is due to debris or bubbles in front of the detector. The signal may be used if peaks remain below the saturation limit of 16,000 units.
 - 10.6.2.2 Improper loading may be visualized. Wait until the primer peaks emerge, before deciding to abort a run.
 - 10.6.3 To monitor signals from all lanes of the assay, expand the display.
 - 10.6.3.1 Highlight all assays and select "View Selected Group". A "Selected Assays View" of the run progress will appear. The "Selected Assays View" window displays signals from all lanes of the assay in real time. Sample signal should appear 10 ½ to 11 minutes after the run began. Large primer peaks appear, followed by sequence signal. Preliminary sequence quality can be evaluated real time. The run may be terminated if poor signal or other problems are observed.

- 10.6.3.2 After 2-5 minutes of signal peak collection, evaluate signal peak “deltas”: the difference between minimum and maximum peak height for each nucleotide/primer pair.
 - 10.6.3.3 Double click and select an area of peaks in the “Run View” screen.
 - 10.6.3.4 Select “Tools/ Inspector/ Selection” to display a table of values for peak minimum, maximum and delta.
 - 10.6.3.5 Peak deltas should have values greater than 1000 units. Flat top peaks indicate signal saturation due to overloading and should be repeated. Peak delta values of less than 700 units may result in poor base calling. If the peak delta value is too low, and the sample loading for that lane was in question, consider re-running the gel.
 - 10.6.3 .6 “Selected Assays View” window can be minimized, closed or left open. DO NOT “CLOSE” GEL RUN WINDOW. THIS WILL TERMINATE THE RUN.
- 10.7 Completion of the electrophoresis and detection run
- 10.7.1 The electrophoresis and detection will automatically terminate after the selected run time has elapsed. The VGI OpenGene™ software performs automated sequence alignment and base calling for each of the primer strands. When the run has completed, the Tower will indicate “Disconnected”.
 - 10.7.2 Close and save the run. Close any associated assay view windows.
 - 10.7.3 Aspirate and discard the buffer from the reservoirs, remove the spent MicroCel™ cassette, discarding in a glass disposal box. Blot buffer chamber reservoirs dry with gauze if another electrophoresis run will follow. Proceed to sequence evaluation.
- 10.8 To abort or terminate a run before completion:
- 10.8.1 Select the specific Run.
 - 10.8.2 Select “Sequencer Control” tab.
 - 10.8.3 Select “STOP.”
 - 10.8.4 At ALERT prompt select “Stop run”. Message box will indicate, “Run terminated by user. Heater set to idle. Voltage and laser power turned off.”
 - 10.8.5 At ALERT prompt select “Cancel/Don’t Save/Destroy”.

- 10.8.6 Message box will indicate “Disconnected” and application will return to Run Setup Wizard screen.
- 10.9 Shut down and clean up
 - 10.9.1 Turn off the power to the Towers.
 - 10.9.2 Remove the buffer chambers from the Towers and rinse with water, blot or air dry.
 - 10.9.3 Clean the heating pad, gaskets and Towers and blot dry.
 - 10.9.4 Return the SureFill™ cartridge to 4°C storage.
 - 10.9.5 Clean Gel Toaster™ assembly with water.
 - 10.9.6 Clean and blot dry gel combs and clips.
- 10.10 Gel RunTrouble Shooting: Refer to Trouble Shooting Appendix.
- 11.0 Procedure: Sequence Evaluation
 - 11.1 Review the Assay
 - 11.1.1 In OpenGene™, select Document/ Open/ Assays.
 - 11.1.2 Select the appropriate database.
 - 11.1.3 Select user/ month/ PID, etc from directory.
 - 11.1.4 Highlight appropriate assay files.
 - 11.1.5 Select Multiviewer option/Open.
 - 11.1.5.1 A “multiviewer” window with all the selected files will appear showing the assay name and the sequence chromatogram. Above the chromatogram, a colored bar displays the OpenGene™ base calls for the assay. A pink spike indicates heterozygotes or ambiguous bases in the sequence. A gray-scale bar below the chromatogram indicates the confidence level of the base call. The lighter the color, the higher the confidence level of the base calls. Other assay files can be selected and moved to the top to view the quality parameters of the base calls.
 - 11.2 Evaluate the Assay
 - 11.2.1 Verify that the negative and positive controls for the sample batch are acceptable.

- 11.2.2 Check the over-all sequence quality for each assay. Look for:
- 11.2.2.1 Low noise/ background.
 - 11.2.2.2 Good peak height and signal.
 - 11.2.2.3 No signal saturation.
 - 11.2.2.4 No obvious cross talk.
 - 11.2.2.5 Clean peaks and good resolution
 - 11.2.2.6 Few strong stops.
 - 11.2.2.7 No alignment problems.
 - 11.2.2.8 Complete base calling across the gene.
 - 11.2.2.9 Check for fewer than 7 ambiguous or “heterozygote” base calls between bases 30-60 and between bases 240-270, with good peak resolution.
 - 11.2.2.10 If these conditions are satisfied, proceed to Sequence Evaluation. Refer to the trouble-shooting appendix if necessary.
 - 11.2.2.11 If the specimen fails to produce adequate signal for analysis, repeat processing to rule out analytic error. If the specimen continues to fail after repeat processing, notify the Director. Recollection may be indicated by study protocol and viral load. Numerous pre-analytic factors may contribute to the failure: actual viral load, non-compliance with enrollment criteria, specimen characteristics including anticoagulant, storage or labeling errors, mutations at the primer sites, patient infected with HIV-2 or HIV-1 non-clade B isolates. Report to the clinic as instructed by the Director, e.g.:
 - Unable to perform genotyping: specimen failed to sequence. Re-collect and resubmit if indicated.

OR

 - Report as directed. Document the communication to the clinic by Fax or e-mail. File the documentation of the report to the clinic with the Gel Run log in the appropriate notebook in lieu of the genotype report.

11.3 Sequence Evaluation

- 11.3.1 After the assay quality has been assessed, select the sense and anti-sense assays for one of the protease primer pairs, and all of the reverse transcriptase assays.
- 11.3.2 Use the GeneLibrarian™ menu to align the individual assays, yielding the HIV-1 sequence for the target regions.
- 11.3.2.1 Select “GeneLibrarian™/ Search and Combine”. GeneLibrarian™ will process the selected assays and generate a case working-window. The aligned sequence of the sample will be displayed, Row Q. This is compared to the wild-type HIV-1 sequence, LAV-1, row “W.” The individual assays used to form the sample sequence are identified by numerals. Differences in the sample sequence relative to the wild-type sequence are highlighted in green. GeneLibrarian™ allows base calls to be reviewed and edited. Double clicking on the consensus sequence row Q displays the chromatogram associated with the bases called at that location. Upon review of the chromatograms, the base called in the consensus sequence may be accepted or edited at the reviewer’s discretion.
- 11.3.2.2 Review all resistance codons, all ambiguous bases, all bases which differ from wild type, and all single stranded base calls.
- 11.3.2.3 Record case number generated.
- 11.3.2.4 Review and verify assay information.
- 11.3.2.5 Review all resistance codons, all ambiguous bases, all bases differing from wild type and all single stranded base calls. Edit as required.
- 11.3.2.6 If the assays were properly evaluated there should be few problems with the case interpretation. Gaps and areas of single strand calls indicate that the sequence information from the assay was of poor quality and rejected by the GeneLibrarian™ application.
- 11.3.2.7 Repeat electrophoresis and detection may resolve these problems. If a good quality sequence is not obtained upon repeat electrophoresis, repeat CLIP™ sequencing, or RT-PCT and CLIP™ may be indicated.
- 11.3.2.8 Mutation calls should be confirmed on both strands. The reviewer should evaluate discordant calls on forward and

reverse strands. In most situations GeneLibrarian™ will interpret discordant strands based on the sequence quality. Any automatic base call may be edited at the discretion of the reviewer.

- 11.3.3 Save the case after completion of editing and review.
- 11.3.4 Proceed with genotype interpretation, reporting and quality control. Refer to: HIV-1 Genotype Reporting and Quality Control

12.0 Procedure: File Management

- 12.1 Periodically compress all completed run, assay, and case files. Store as archived files and copy to ZIP disks as a back up.
- 12.2 Files cannot be accessed in compressed format must be decompressed before viewing. Use the FSDB Manager to create and re-index database after decompression.
- 12.3 Refer to the Bayer User's Manuals.

13.0 Attachments

- 13.1 Specimen Log
- 13.2 Specimen Tracking Form
- 13.3 Sample Processing Log
- 13.4 Bench-top Guide: VGI RT-PCR/CLIP Solutions
- 13.5 CLIP™ Sequencing Template Diagram
- 13.6 Thermal Cycler Run Log
- 13.7 Gel Run Log
- 13.8 Master Run Log
- 13.9 Genotype Report Facsimile Transmittal Sheet

14.0 Appendix

- 14.1 Trouble Shooting Appendix

15.0 References

- 15.1 Center for Disease Control. 1988. Update: Universal Precautions for Prevention of Transmission of Human immunodeficiency Virus, Hepatitis B Virus and Other Bloodborne Pathogens in Healthcare Settings. MMWR, 37:337-382, 387, 388.
- 15.2 TRUGENE™ HIV-1 Genotyping Kit VG30210 Package Insert.
- 15.3 Sambrook, J., E.F. Fritsch, T. Maniatis. 1989. Molecular Cloning, A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, 6.2-6.7.
- 15.4 Bayer User's Manuals

Procedure: Bayer TRUGENE HIV-1 Genotyping Procedure

Prepared by: ACTG Laboratory Technologists Committee

Preparation Date: 01 June 2004

Date Implemented into the Laboratory: _____

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Reviewed by: _____ Date: _____

Supersedes Archived Protocol:

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