1. PRINCIPLE

Accurate detection of peak height and position is crucial to the total quality assurance of the VGI sequencing system. M13 is a well-characterized, "well behaved" phagemid clone used in a variety of molecular applications. Periodic calibration with the VGI M13 Panel ensures performance of the integrated Long-Read Tower Sequencers. The M13 Calibration Panel consists of prepared sequences labeled with the same fluorescent dyes used for patient samples. The M13 test dyes are mismatched so that the forward sequence dye and the reverse sequence dye for each nucleotide are detected in separate lanes, rather than in the same lane as they are for patient assays. In this way, each base/dye combination is evaluated. The M13 panel tests the performance of the electrophoresis unit, laser detectors and integrated analysis system for sequence determination of known samples with expected signal height, retention and base sequence. VGI participates in the evaluation of the calibration panel, providing feedback to the user in order to maintain optimum performance.

2. PRECAUTIONS

2.1. 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 UCHSC waste disposal policy.

2.2. Formamide

The M13 calibration panel contains formamide. 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 biohazardous waste stream. Refer to the Chemical Hygiene Plan and the Policy for Specimen Collection and Handling.

2.3. 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.

2.4. 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.

2.5. UV Light Source

A UV light source in the Gel Toaster™ to 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. REAGENTS AND EQUIPMENT

3.1. VGI M13 stocks, VGI Part #PA0700845. Store at -20°C.

3.1.1. Tube #1: T Cy™ 5.0 and A Cy™ 5.5
3.1.2. Tube #2: G Cy™ 5.0 and C Cy™ 5.5
3.1.3. Tube #3: C Cy™ 5.0 and G Cy™ 5.5
3.1.4. Tube #4: A Cy™ 5.0 and T Cy™ 5.5

3.2. 0.2 ml PCR tubes or strips with lids and tray.

3.3. Thermal cycler and additional equipment and supplies needed to prepare, load and run sequencing gels as for the patient samples. Refer to HIV-1 TRUGENE™ genotyping protocol.

4. STANDARD/CONTROL

4.1. Perform calibration quarterly and whenever performance is suspect.

4.2. Perform calibration when changing suppliers of TBE.

4.3. Run Time:

4.3.1. Run time for the region between the target G and C peaks described below: 19-25 minutes for MicroCel™ cassettes.

4.3.2. If out of range, verify gel and buffer quality. Repeat assay. Request service if the performance is substandard.

4.4. VGI Analysis: Follow instructions from the manufacturer.

4.5. Notify the Director and co-workers if performance is out of range. Testing of patient samples may continue at the discretion of the Director.

5. PROCEDURE

5.1. Prepare M13 Reagent

5.1.1. Thaw the stock M13 tubes, vortex briefly and hold on ice.

5.1.2. Prepare a gel-loading tray with aliquots of M13 tubes 1-4 adequate for delivery of 1.5µl for each lane of each tower to be tested. Aliquot M13 dyes to a row of 8 tubes: 1,2,3,4,1,2,3,4 to mimic the load timing of patient samples. For example, tube #1 will load to lanes 1,5,9,13; tube # 2 will load to lanes 2,6,10,14, etc. To calibrate 4 towers, aliquot 15µl of each M13 dye to the appropriate tube in the tray:

M13 PCR Tray Layout							
A	B	C	D	E	F	G	H
15µl Tube #1	15µl Tube #2	15µl Tube #3	15µl Tube #4	15µl Tube #1	15µl Tube #2	15µl Tube #3	15µl Tube #4

5.1.3. Hold M13 stock on ice or place at 4°C. Do not refreeze.

5.2. Denature the M13 reagents: Cap the PCR tubes and denature the M13 aliquots in using the thermal cycler "Denature" program, 3 minutes at 85°C. Store on ice protected from light.

5.3. Electrophoresis and Detection

5.3.1. Set up data files:

5.3.1.1. In OpenGene application select "Tools/ Run Setup Wizard."

5.3.1.2. Select database: M13 Trials.

5.3.1.3. Select Setup: M13 2-Dye.

5.3.1.4. Click "Create Run".

5.3.1.5. Select "Assay Information" tab.

5.3.1.6. Select any set of four lanes from bottom "Cy™ 5.0" row.

5.3.1.7. Verify Dye = Cy™ 5.0 and Region: M13.

5.3.1.8. Select all Cy™ 5.0 lanes (bottom row).

5.3.1.9. At lower left select "Unassign." Bottom row will go blank.

5.3.1.10. At right side of screen select and reverse the base ordering to read TGCA from left to right.

5.3.1.11. Select "ALT-S" to save.

5.3.1.12. Connect to the Long-Read Towers as for routine run. All run parameters (time, temperature, voltage, laser power, etc.) should be set as in patient testing.

5.4. Prepare and load gels

5.4.1. When the Towers are ready, prepare and load gels as using the same technique as for patient testing. Load the M13 reagents to each assigned lane, repeating for each tower.

Lane:	***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***
M13 tube #:	1	2	3	4	1	2	3	4
Cy™5.0	T	G	C	A	T	G	C	A
Cy™5.5	A	C	G	T	A	C	G	T
Lane:	***9***	***10***	***11***	***12***	***13***	***14***	***15***	***16***
M13 tube #:	1	2	3	4	1	2	3	4
Cy™5.0	T	G	C	A	T	G	C	A
Cy™5.5	A	C	G	T	A	C	G	T

5.5. Evaluate the Calibration Run

5.5.1. Preliminary assessment and modification.

5.5.1.1. All sequences should appear to be identical.

5.5.1.2. Open “Raw Data Viewer” from the run window and verify that all Cy 5.0 and Cy 5.5 lanes have signal and that baseline is OK.

5.5.1.3. Verify that there is no “cross-talk” (minor peaks coinciding with signal from other lanes) between lanes and that signal peaks are not saturated. If present, repeat gel with decreased volume of sample. Contact VGI if the problem is not resolved upon repeat.

5.5.1.4. Close raw data viewer.

5.5.1.5. Open the assays in the “multiviewer” window. Find the first double G in the sequence followed by TCGA. Delete all base calling preceding the double G.

5.5.1.6. Repeat for all assays in the run.

5.5.1.7. Select “ALT-S” to save changes.

5.5.2. Determine run time for M13 calibration sequence:

5.5.2.1. Verify acceptability of peak resolution and clarity for all assays, using the standard criteria for assays of patient samples.

5.5.2.2. Single click on center of first G peak of G doublet. Scroll to C peak centered between 5 T’s to left and 4 T’s to right near base 270-290 in the middle of the sequence. Hold “SHIFT” key and single click on the center of the C peak. This will result in a gray-highlighted area between the selected G and C peaks.

5.5.2.3. Select “Tools/ Inspector/ Selection” and determine the duration of the gel run for this region. This interval should be about 19-25 minutes for MicroCel™ cassettes run under standard conditions.

5.6. Compress and copy assay files. Send to VGI for analysis

5.7. Record calibration results in VGI Gel Run / QC files. Include the duration of the run for each set or 4 lanes, corresponding to the lanes used for each primer pair, i.e., record data for lanes 1-4, 5-8,9-12,13-16.

6. ATTACHMENTS

6.1. Gel Run Log

7. REFERENCES

7.1. CRL VGI HIV-1 TRUGENE™ Genotyping Procedure

7.2. VGI: Calibrating Your Equipment (Memorandum)