QUANTIFICATION OF CELLULAR DNA, TOTAL HIV DNA, AND INTEGRATED HIV DNA BY QPCR

I. Quantification of Cellular DNA

Follow this protocol after purifying DNA as described in Appendix A. We prepare our master mixes in large batches and freeze aliquots at -20°C. See Appendix C for information on master mix preparation.

1. Quantify your DNA samples by spectrophotometry. If needed, appropriately dilute a fraction of the sample so that the resulting DNA concentration falls within the standard curve range (i.e. 300-3,000 cells per uL).
2. Measure each sample at three different dilutions in triplicate; each sample requires 9 wells. Diluting the sample helps to check for PCR inhibitors; a 2-fold dilution should result in 2-fold reduction in the number of cells per well calculated by the qPCR instrument.
3. Thaw Albumin Master Mix (see Appendix C for recipe). At a minimum, thaw a volume of: (15 µL)*((# of samples x 9) + 4). This accounts for volume loss that may occur during pipetting.
4. Once thawed, add Taq at a ratio of 1:100 to the master mix. Mix by inverting several times.
5. Pipette 15 µL of master mix into the desired wells of a PCR plate.
6. Add 15 µL of each DNA sample and DNA standard (Table 1) to appropriate wells.

   6.1. Standard curve for cellular DNA

   We use serial dilutions of human DNA to generate a standard curve for our cellular DNA measurements. The DNA is quantified by repeat measurements on a nanospectrophotometer, which are then averaged.

Table 1. Cellular DNA Dilutions

Concentration (Cells/15 µl)	Dilution	Well Locations	Volume from previous dilution (µl)	Volume H_2O (µl)
50,000	N/A	A1, A2	N/A	N/A
25,000	1:2	B1, B2	100	100
12,500	1:2	C1, C2	100	100
6,250	1:2	D1, D2	100	100
3,125	1:2	E1, E2	100	100
1562.5	1:2	F1, F2	100	100
781.25	1:2	G1, G2	125	125
0 (NTC - Water)	N/A	H1, H2	N/A	N/A

7. After adding all samples to the appropriate wells, seal the plate with qPCR film.
8. Spin the plate in a centrifuge to collect any droplets.
9. Place the plate in an ABI 7500 Fast qPCR machine* and cycle it under the conditions listed in Table 2.

Table 2. Cellular DNA qPCR Cycle Conditions.

Albumin	°C	Time
Denaturation	95	2 minutes
Cycling (40 cycles)	95	10 seconds
	60	30 seconds, Signal acquisition

*Other qPCR machines are suitable, but optimal thermocycler parameters will need to be determined.

10. Analyze the results with the ABI software to generate the quantity of cells per well.
11. The number of cells per µL of the sample stock solution, n, can be calculated by dividing the reported quantity, q, by 15 and then multiplying by the dilution factor, d. This calculation should be performed for each of the 9 wells, and then the 9 values can be averaged.

$$n = \frac{q \times d}{15}$$

II. Pre-amplification, Total HIV Measurements

Calculate the cellular concentration of your sample DNA solution using the albumin assay (Part I) prior to making HIV measurements. We prepare our master mixes in large batches and freeze aliquots at -20°C. See Appendix C for information on master mix preparation.

1. Measure each sample at three different dilutions in triplicate; each sample requires 9 wells. Diluting the sample helps to check for PCR inhibitors; a 2-fold dilution should result in 2-fold reduction in the number of HIV copies per well calculated by the qPCR instrument. We generally measure samples at 300,000; 150,000; and 75,000 cells per well in triplicate.
2. Thaw the Total HIV pre-amplification master mix. At a minimum, thaw a volume of: (25 µL)*((# of samples x 9) + 4). This accounts for volume loss that may occur during pipetting.
3. Add Taq Polymerase at a ratio of 1:50 to master mix volume.
4. Mix by inverting several times.
5. Distribute 25 µL of master mix into the appropriate wells.
6. Add 25 µL of each DNA sample and DNA standard (Table 3) to the appropriate wells.

   6.2 Standard curve for Total HIV DNA

   We use serial dilutions of an integration standard (I.S.) created in our lab as a standard curve for our total HIV measurements. The method of how this standard was created is detailed in a previous publication[1]. Plasmid standards are also suitable for measurements of total HIV, but for simplicity, we choose to use the "integration standard" for both our Total HIV and Integrated HIV measurements.

Table 3. Total HIV Dilutions

Concentration (HIV copies/25 µl)	Dilution	Well Locations	Volume from previous dilution (µl)	Volume H_2O (µl)
2,000	N/A	A1, A2	N/A	N/A
1,000	1:2	B1, B2	100	100
500	1:2	C1, C2	100	100
250	1:2	D1, D2	100	100
125	1:2	E1, E2	100	100
62.5	1:2	F1, F2	100	100
31.25	1:2	G1, G2	125	125
0 (NTC - Water)	1:2	H1, H2	125	125

7. After adding all samples to the appropriate wells, seal the plate with PCR film.
8. Spin the plate in a centrifuge to collect any droplets.
9. Place the plate in an Eppendorf nexus PCR machine and cycle it under the conditions listed in Table 5.

III. Pre-amplification, Integrated HIV Measurements

Calculate the Total HIV Concentration (Part II) prior to beginning. We prepare our master mixes in large batches and freeze aliquots at -20°C. See Appendix C for information on master mix preparation.

1. Thaw Integrated HIV Master Mix and Linear Gag Master Mix. At a minimum, thaw a volume of 2300 µL Integrated HIV Master Mix and 250 µL of Linear Gag Master Mix.
2. Add Taq Polymerase at a ratio of 1:50 to master mix volume.
3. Mix by inverting several times.
4. Using the results of the Total HIV measurement, determine the volume of sample required to distribute 12 copies of HIV per 25 µL volume. Create 1850 µL of this solution using water as your diluent and vortex to mix. This is your first dilution (Dilution 1) of the sample. *12 copies of HIV per well is targeted because we find that this results in approximately 65% positive wells. The range of 60-90% positive wells has implications in the Poisson model for our sample size.*

   4.2 For instance, if your Total HIV measurements show that a sample contains 1200 HIV copies per million cells, you would distribute 10,000 cells per well in a volume of 25 µL, corresponding to 12 copies of HIV per well. You would generate 1850 µL of a 400 cells per µL solution.
5. Pipette 240 µL of Dilution 1 into a new tube. Add 240 uL of Linear Gag master mix to the same tube and vortex gently to mix. Distribute 50 µL of the Linear Gag-Dilution 1 mixture to the appropriate wells (see Table 4, 8 replicate wells).
6. Pipette 875 µL of Dilution 1 into a new tube. Add 875 uL of Integrated HIV master mix to the same tube and vortex gently to mix. Distribute 50 µL of the Integrated-Dilution 1 mixture to the appropriate wells (see Table 4, 32 replicate wells).
7. To create your second dilution (Dilution 2) of the sample, add 670 µL of Dilution 1 to 670 µL of water in a new tube. Add 1340 µL of Integrated HIV master mix to the same tube and mix by vortexing gently. Distribute 50 µL of the Integrated-Dilution 2 mixture to the appropriate wells (see Table 4, 48 replicate wells).
8. Add 25uL of Integrated HIV master mix to the wells in Column 1 (A1, B1, C1, etc).
9. Add the standards, as described in Table 4, to wells in Column 1.

   9.2 Our Integration Assay uses probability theory following the Poisson distribution[2,3,4] to quantify proviral HIV without the use of a standard curve. While typically used in a dPCR setting, the Poisson distribution can be applied to a 96-well plate. We include a small amount of standard on our plates to test thermocycler conformity from run to run as well as check for PCR inhibitors that may be present in the sample DNA. Furthermore, we include 8 wells of master mix that contain only the Gag primer, which allows for linear amplification during cycling. Linear amplification after 40 cycles of pre-amplification can be detected by subsequent qPCR, and so the Linear Gag wells act as a background control for potential unintegrated HIV targets.

Table 4. Integrated HIV Dilutions

Master Mix	Integration Standard 600 copies/12.5 µl (uL)	Sample DNA Dilution 1 (uL)	Sample DNA Dilution 2 (uL)	Water (µL)	Well Locations
Integration	12.5	0	0	12.5	A1, B1
Integration	12.5	12.5	0	0	C1, D1
Integration	0	0	0	25	E1, F1, G1, H1
Integration	0	25	0	0	Columns: 3, 4, 5 Wells: 6A, 6B
Integration	0	0	25	0	Wells: 6E, 6F, 6G, 6H Columns: 7,8,9,10,11,12
Linear Gag	0	25	0	0	A2, B2, C2, D2, E2, F2, G2, H2

10. After adding all samples to the appropriate wells, seal the plate with PCR film.
11. Spin the plate in a centrifuge to collect any droplets.
12. Place the plate in an Eppendorf Nexus PCR machine* and cycle it under the conditions listed in Table 5.

Table 5. Total and Integrated HIV PCR Cycle Conditions

TOTAL HIV	°C	Time (mm:ss)	Integrated HIV	°C	Time (mm:ss)
Denaturation	95	2:00	Denaturation	95	2:00
Cycling (12 cycles)	95	0:15	Cycling (40 cycles)	95	0:15
	64	0:45		56.2	0:45
	72	1:00		72	3:30
Elongation	72	10:00	Elongation	72	10:00
Storage	10	Hold	Storage	10	Hold
Eppendorf Standard Mode Ramp Rate: 1.5°C/second			Eppendorf Standard Mode Ramp Rate: 1.5°C/second		

*Other thermocyclers are suitable, but optimal cycling parameters will need to be determined

IV. qPCR Amplification; Total HIV and Integrated HIV Measurements

Samples should be pre-amplified prior to quantifying by qPCR; see Parts II and III. We prepare our master mixes in large batches and freeze aliquots at -20°C. See Appendix C for information on master mix preparation.

1. Thaw RU5 qPCR master mix. At a minimum, thaw a volume of: (15 µL)*(# of wells to transfer + 4).
2. Once thawed, add Taq at a ratio of 1:100 to the master mix. Mix by inverting several times.
3. Pipette 15 µL of master mix into the desired wells of a qPCR plate.
4. Spin completed pre-amplification plate to collect droplets.
5. Pipette up and down to mix the solution in the pre-amplification plate. Transfer 15 µL from each well of the pre-amplification plate into the qPCR plate.
6. After transferring, seal the plate with qPCR film.
7. Centrifuge the plate to collect any droplets.
8. Place the plate in an ABI 7500 Fast qPCR machine* and cycle it under the conditions listed in Table 6.

Table 6. Total and Integrated HIV qPCR Cycle Conditions

RU5 (Total & Integrated HIV)	°C	Time
Denaturation	95	2 minutes
Cycling (40 cycles)	95	10 seconds
	60	30 seconds, Signal acquisition

*Other qPCR machines are suitable, but optimal thermocycler parameters will need to be determined.

9. For Total HIV measurements, analyze the results with the ABI software to calculate the number of HIV copies per well. The number of HIV copies per million cells of the sample stock solution can be calculated by dividing the reported quantity by the number of cells in the well and then multiplying by 1,000,000. This calculation should be performed for each of the 9 wells, and then the 9 values can be averaged.
10. For integrated HIV measurements, see section V.

V. Analysis of Integrated HIV Measurements

1. Adjust the threshold value so that CT values are reported for the linear phase of the Linear Gag wells. Omit any wells that intersect with the threshold during their plateau phase. On our instruments, we generally set the threshold between 0.01 and 0.02. It is important to keep the threshold value the same for both Linear Gag and Integrated HIV master mixes, although the value can be changed from plate to plate.

2. Average the CT values of the Linear Gag wells. It is likely that not all of the wells will have a CT value and will be marked as Undetermined; this is expected.

3. Calculate the standard deviation of the CT values of the Linear Gag wells.

4. Determine your CT value cut-off (baseline) by subtracting 2 times the value of the standard deviation from the average CT of Linear Gag wells. Any Integrated HIV wells falling above this value should not be counted in the subsequent analysis because they likely do not contain integrated HIV (amplification could be coming from unintegrated HIV).

5. Count the number of positive wells (those with a CT value less than the established baseline) for each dilution assayed for Integrated HIV. It is important that 60-90% of the wells are positive for each dilution, as error increases outside of this range.

6. Use the following formula to calculate the number of integrated HIV copies per million cells:

$$y = 10{,}000{,}000 \times \frac{-\ln\left(1 - \left(\frac{x}{100}\right)\right)}{z}$$

Where,
y = number of integrated HIV copies per million cells
x = % positive wells
z = number of cells per well

Appendix A: DNA Purification Protocol: Gentra Puregene Cell Kit (Qiagen)

This protocol was adopted from the Gentra Puregene Kit Handbook, and was optimized for the purification for 50 million cells. DNA from a smaller number of cells can be purified by scaling buffer volumes proportionally and using the appropriate tube sizes, though optimization may be necessary. Solutions used in this protocol are provided with the Gentra Puregene Cell Kit (Qiagen, #158745).

1. Spin $50x10^6$ cells in a 50 mL conical, 800 x g for 10 minutes at 37°C in a swing bucket rotor. Place a fixed angle rotor in the centrifuge and begin chilling to 4°C.
2. Pour off supernatant. Re-suspend the pellet in the small amount of remaining supernatant.
3. Add 6 mL cell lysis solution to the cells, then pulse vortex at full speed 6 times.
4. Add 30 uL RNase A, and mix by inverting 10 times.
5. Incubate in a water bath at 37°C for 45 minutes. Add weights to the tubes so that they stay submerged.
6. Place tubes on ice for 5 minutes.
7. Add 2 mL of protein precipitation solution.
8. Mix by pulse vortexing at full speed 15 times.
9. Spin the 50 mL conicals in a fixed angle rotor at 19,000 x g for 15 minutes. All of the white precipitate should be spun down in a tight pellet.
10. While the samples spin, add 6 mL of isopropanol to new 50 mL conicals (1 for each sample). Chill a microcentrifuge to 4°C
11. When the spin is complete, pour the supernatant into the tube containing the isopropanol. Invert 50 times, insuring to wash all sides of the tube by rotating. A large, white, DNA precipitate should form.
12. Add 900 uL of 70% ethanol to a 1.5 mL snap-cap tube (1 for each sample).
13. Using a p1000, remove the DNA precipitate from the isopropanol, and place it into the 70% ethanol. Transfer as little isopropanol as possible.
14. Spin the 1.5 mL tube at max (~17,000 x g) for 1 minute at 4°C.
15. Pour off the ethanol, being careful to retain the DNA pellet. Pipette off as much of the remaining ethanol as possible using the p200.
16. Quick-spin the tubes to collect the remaining ethanol. Using the p20, pipette off remaining ethanol.
17. Let DNA pellets dry for 30 minutes.
18. Turn on heated shaker to 65°C.
19. Check to be sure no ethanol remains by flicking the tubes.
20. If pellets are free of ethanol, add 500 uL of 10 mM Tris-HCl, pH 8.0
21. Place tubes in a heated shaker and adjust program to 37 C for ~24 hours, 400 rpm. Do not heat DNA at 65°C for over 1 hour; it causes extreme fragmentation of the DNA.
22. The following day, vortex the samples and check to see how well the DNA pellet has been resuspended. You will no longer see a white pellet, but make sure the solution doesn't appear non-homogenous. The easiest way to check for this is to flick the tube and watch the air bubbles, which should rise freely if the solution is homogenous.
23. If your solution is non-homogenous, you can add more buffer or wait longer.
24. Once DNA solution is mostly homogenous, shear DNA by passing it through a 25 gauge needle 10 times (draw it up 10 times, it will technically pass the needle 20

times). This results in a homogenous DNA solution of molecular weight of >20 kb. I've never mechanically sheared DNA that was still goopy, so I can't comment on its effect on DNA molecular weight.

25. DNA solution can now be quantified by qPCR and used for other applications.

Appendix B: Probes and Primers sequences

Cellular DNA Primers and Probe
Albumin F: 5'-GCTGTCATCTCTTGTGGGCTGT3'
Albumin R: 5'-AAACTCATGGGAGCTGCTGGTT-3'
Albumin Probe: 5'-6FAM/CCTGTCATGCCCACACAAATCTCTCC/BHQ1-3'

Total HIV Primers, 1st PCR
RU5 F: 5'-TTAAGCCTCAATAAAGCTTGCC-3'
RU5 R: 5'-GTTCGGGCGCCACTGCTAGA-3'

Integrated HIV Primers, 1st PCR
Alu F: 5'-GCCTCCCAAAGTGCTGGGATTACAG-3'
SK431 R: 5'-TGCTATGTCAGTTCCCCTTGGTTCTCT-3'

RU5 qPCR Primers and Probes
RU5 F: 5'-TTAAGCCTCAATAAAGCTTGCC-3'
RU5 R: 5'-GTTCGGGCGCCACTGCTAGA-3'
Yun's Probe: 5'-6FAM/CCAGAGTCACACAACAGACGGGCACA/BHQ1-3'

Appendix C: 2x Master Mix Recipes

Pre-Amplification 2x Master Mixes

Total HIV	[ ]	**Integrated HIV**	[ ]
Taq Buffer Invitrogen	2x	Taq Buffer Invitrogen	2X
$MgCl_2$ Invitrogen	11mM	$MgCl_2$ Invitrogen	3mM
dNTPs	600µM	dNTPs	400µM
RU5 F	600nM	Alu F	200nM
RU5 R	600nM	Gag R	1.2µM
Taq Invitrogen (5U/µl)	2.5U	Taq Invitrogen (5U/µl)	2.5U

qPCR 2x Master Mixes

RU5	[ ]	**Cellular DNA**	[ ]
Invitrogen 10x PCR Buffer	2x	Invitrogen 10x PCR Buffer	2x
$MgCl_2$ Invitrogen	11mM	$MgCl_2$ Invitrogen	7mM
dNTPSs	600µM	dNTPs	400µM
RU5 F	600nM	Albumin F	2µM
RU5 R	600nM	Albumin R	2µM
Yun's Probe	400nM	Albumin Probe	400nM
ROX	1:100	ROX	1:100
Taq Invitrogen (5U/µl)	0.75 U	Taq Invitrogen (5U/µl)	0.75 U

Appendix D: Reagents

- Sterile water – *Sigma Aldrich, Cat. No. W4502*
- Platinum Taq DNA Polymerase – *ThermoFisher, Cat. No. 10966*
- dNTP Set – *ThermoFisher, Cat. No. 10297*
- $MgCl_2$ – *ThermoFisher*, *Cat. No. AM9530G*
- PCR plate sealing film – *Sarstedt, Cat. No. 95.1994*
- 96-well PCR Plates – *Denville Scientific, Cat. No. C18096-10*
- qPCR plate sealing film, optically clear – *ThermoFisher Cat. No. 4311971*
- 96-well qPCR Plates – *Denville Scientific, Cat. No. C18081-105*
- Primers and Probes – *Integrated DNA Technologies*
- Gentra Puregene Cell Kit – *Qiagen, Cat. No. 158745*

Appendix D: References

1. Liszewski, M. K., et al. (2009). "Detecting HIV-1 integration by repetitive-sampling Alu-gag PCR." Methods **47**(4): 254-260.
2. Sykes, P. J., et al. (1992). "Quantitation of targets for PCR by use of limiting dilution." Biotechniques **13**(3): 444-449.
3. Sedlak, R. H. and K. R. Jerome (2013). "Viral diagnostics in the era of digital polymerase chain reaction." Diagn Microbiol Infect Dis **75**(1): 1-4.
4. De Spiegelaere, W., et al. (2014). "Quantification of integrated HIV DNA by repetitive-sampling Alu-HIV PCR on the basis of poisson statistics." Clin Chem **60**(6): 886-895.