PROTOCOL FOR: Fragmentation of genomic DNA for Droplet Digital PCR using the QIAshredder saves time and results in higher copies compared to restriction digestion with RsaI or BsaJI

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REAGENTS:

Leukoreduction filters from blood donors (Blood Systems Research Institute, San Francisco, CA, U.S.A.)
Ficoll-Paque PLUS (G.E. Healthcare, Pittsburgh, PA, U.S.A.)
Dulbecco's Phosphate Buffered Saline (DPBS) 1x (Gibco/Life Technologies, Grand Island, NY, U.S.A.)
RPMI Medium 1640 with L-glutamine (Gibco/Life Technologies, Grand Island, NY, U.S.A.)
Penicillin 10,000U/ml + Streptomycin 10,000µg/ml (100x) (Gibco/Life Technologies, Grand Island, NY, U.S.A.)
L-Glutamine 200mM (100x) (Gibco/Life Technologies, Grand Island, NY, U.S.A.)
Fetal Bovine Serum (Sigma, St. Louis, MO, U.S.A.)
8E5/LAV Cells (NIH AIDS Reagent Program, Bethesda, MD, U.S.A.)
DNase, RNase free water (Sigma, St. Louis, MO, U.S.A.)
Trireagent (Molecular Research Center, Inc., Cincinnati, OH, U.S.A.)
guanidine thiocyanate (Sigma, St. Louis, MO, U.S.A.)
sodium citrate (Sigma, St. Louis, MO, U.S.A.)
Trizma base (Sigma, St. Louis, MO, U.S.A.)
QIAgen DNA Blood Mini Kit (QIAgen, Valencia, CA, U.S.A.)
QIAshredder spin columns (QIAgen, Valencia, CA, U.S.A.)
RsaI with NEB Buffer 4 (New England BioLabs, Ipswich, MA, U.S.A.)
BsaJI with Smart Cut Buffer (New England BioLabs, Ipswich, MA, U.S.A.)
UltraPure Agarose (Invitrogen/Life Technologies, Green Island, NY, U.S.A.)
10xTBE Buffer (Promega)
SYBR Green I Nucleic Acid Gel Stain, 10,000x (Sigma, St. Louis, MO, U.S.A.)
Lambda DNA/HindIII Fragments (Invitrogen/Life Technologies, Green Island, NY, U.S.A.)

High DNA Mass Laddder (Invitrogen/Life Technologies, Green Island, NY, U.S.A.)
E-Gel 96 High Range DNA Marker (Invitrogen/Life Technologies, Green Island, NY, U.S.A.)
BlueJuice Gel Loading Buffer (Invitrogen/Life Technologies, Green Island, NY, U.S.A.)
TaqMan Copy Number Reference Assay TERT, 3000rxns (4403315; Applied Biosystems/Life Technologies, Grand Island, NY U.S.A.)
Custom primers for HIV LTR (Sigma, St. Louis, MO, U.S.A.)
Custom probe for HIV LTR (with 5'FAM and 3'BHQ) (Sigma, St. Louis, MO, U.S.A.)
2x ddPCR mix (Bio-Rad, Hercules, CA, U.S.A.)
Droplet Generator DG8 Cartridges (Bio-Rad, Hercules, CA, U.S.A.)
ddPCR Droplet Generation Oil (Bio-Rad, Hercules, CA, U.S.A.)
Droplet Generator DG8 Gasket (Bio-Rad, Hercules, CA, U.S.A.)
Twin.tec PCR plate 96, green, semi-skirted, 250ul (Eppendorf, Hauppauge, NY, U.S.A.)
Pierceable Foil Heat Seal (181404; Bio-Rad, Hercules, CA, U.S.A.)

PROCEDURES:

Isolation of Peripheral Blood Mononuclear Cells (PBMC): PBMC were isolated from leukoreduction filters (obtained via the Blood Systems Research Institute) via centrifugation on a Ficoll-Paque (G.E. Healthcare) density cushion, as described previously[9]. Freshly-isolated PBMC (in phosphate buffered saline) were aliquoted in tubes with $5x10^6$ cells and frozen at -80C for subsequent nucleic acid extraction.

Culture of 8E5/LAV Cells: 8E5 cells (obtained through the NIH AIDS reagent program) were cultured in RPMI 1640 (Gibco/Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Sigma), penicillin 100U/ml + streptomycin 100µg/ml (Gibco/Life Technologies), and 2mM L-glutamine (Gibco/Life Technologies). After 6 passages, 8E5 cells were aliquoted in 8 tubes, each with $3x10^6$ cells per tube, and frozen at -80C for subsequent nucleic acid extraction.

DNA extraction using Trireagent (Molecular Research Center, Inc.): Total RNA and DNA were extracted according to the manufacturer, except that DNA was isolated using the alternative protocol with back extraction buffer (4M guanidine thiocyanate, 50mM Na citrate, and 1M Trizma base), followed by

precipitation with 500µl of isopropanol, 2 washes with 75% ethanol, and resuspension in 10mM Tris pH7.5.

DNA extraction using the QIAgen DNA Blood Mini Kit: DNA was extracted according to the manufacturer, except that elution was performed with 10mM Tris, pH 7.5.

QIAshredding: One aliquot of each Trireagent or QIAgen DNA extract was fragmented using the QIAshredder according to the manufacturer's instructions. The extracted DNA was pipetted repeatedly to maximize homogeneity, then 50-100µl was removed and loaded onto a QIAshredder spin column, which was spun at maximum speed for 3 minutes. The flow-through was resuspended and pipetted to a fresh labeled tube. DNA concentrations of the QIAshredded and unshredded DNA were measured by UV Spectrophotometry (NanoDrop).

Measurement of DNA Concentration: The DNA concentration of each sample (unshredded or QIAshredded) was measured at least 3 times using the ND-1000 (NanoDrop) according to the manufacturer's instructions.

Preparation of equal concentrations of unprocessed, QIAshredded, and digested DNA: Aliquots of unshredded and QIAshredded DNA were diluted in water to make 50µl with a final concentration of either 300ng per 5µl (60ng/µl) or 1000ng per 5µl (200ng/µl), while another aliquot of unshredded DNA was diluted to an identical concentration in a 50µl restriction digest reaction containing water, 10x buffer, and enzyme. For BsaJI digestion, the DNA was first mixed with water and then heated and quenched (see below) before adding buffer and enzyme.

RsaI digestion: RsaI digestion was performed according to the manufacturer's instructions, with a 50µl reaction containing DNA, 5µl of 10x NEB Buffer 4, and 1µl (10 units) of RsaI (New England BioLabs), incubation for 1h at 37C, and heat inactivation at 65C for 20min.

BsaJI digestion: Extracted DNA was heated to 95C for 10min, then quenched on ice for 5min, then digested according to the manufacturer in a 50µl reaction containing DNA, 5µl of 10x NEB smart buffer, and 1µL of BsaJI (New England BioLabs), with incubation at 60C for 1h and heat inactivation at 80C for 10min.

Agarose gel electrophoresis: A 0.8% (wt/vol) agarose gel was prepared by adding 0.48g of UltraPure Agarose (Invitrogen) to 60ml of 1x TBE (prepared by 1:10 dilution of 10xTBE Buffer (Promega) in water), microwaving to dissolve the agarose, adding 7ul of 1x SYBR Green (prepared by 1:10,000 dilution of 10,000x SYBR Green I Nucleic Acid Gel Stain (Sigma)), and pouring into the tray of a Model B1A gel box (Owl Separation Systems, Inc) with 10-well comb. An additional 5ul of 1x SYBR Green was added to the 1x TBE running buffer. Size markers (ladders) included 1ul (500ng) of Lambda DNA/HindIII Fragments (Invitrogen), 4ul of High DNA Mass Laddder (Invitrogen), or 10ul (100ng) of E-Gel 96 High Range DNA Marker (Invitrogen). Samples and ladders were prepared by combining DNA (1000ng) or ladder (1-10ul, as above) with 1x TBE and 3ul of BlueJuice Gel Loading Buffer (Invitrogen) in a total of 15ul. Electrophoresis was performed at approximately 45V, and serial images were obtained using the Typhoon FLA 7000 imager (General Electric).

Serial dilution of DNA: In order to test a range of copy numbers, and also dilute potential inhibitors, each sample of unprocessed, QIAshredded, or digested DNA was diluted serially in water. DNA at 300ng/5µl was diluted in 5-fold increments (10µl in a total of 50µl) to a concentration of 0.00384ng/5µl, while DNA at 1000ng/5µl was diluted in 2-fold increments down to 15.625ng/5µl.

Preparation of PCR mixes: The primers/probe for TERT were obtained in a 20x mix from Applied Biosystems (TaqMan Copy Number Reference Assay for TERT, 3000rxns, 4403315), while a 20x mix was prepared for the HIV primers/probe so that the final 1x concentration would be 900nM of each primer and 250nM or probe.

DNA inputs were designed to range from 0.00384ng to 300ng (0.6 to 48,000 cell equivalents), 15.625ng to 1000ng (2,500 to 160,000 cell equivalents), or 15.625 to 1800ng (2,500 to 288,000 cell equivalents) per 20µl reaction. Each input mass of each DNA type was tested in replicate. To ensure that we had enough for three 20ul reactions (given possible losses in pipetting), for each input mass of DNA, we prepared enough for 3.5 reactions by preparing 20ul*3.5=70ul of the final reaction mix. Each 70ul had 35ul of 2x ddPCR mix (Bio-Rad) and 3.5ul of 20x primer/probe mix. However, to ensure more equal conditions in all samples, the 2x ddPCR mix and 20x primer/probes (and usually water) were prepared together in a master mix for all samples.

For experiments with a maximum DNA input of 300ng or 1000ng, we prepared a master mix containing 10ul of 2x ddPCR mix, 1ul of 20x primer/probe mix, and 4ul of H20 for every 20ul of final

reaction, and then added 15ulx3.5=52.5ul of this master mix for every 70ul of final reaction mix. We then added 5ul*3.5=17.5ul of DNA at the appropriate concentration (say, 300ng/5ul=60ng/ul), bringing the total to 70ul. This 70ul was then mixed, and 20ul aliquots were used in three separate wells to make droplets. To achieve an input of 1800ng, we first prepared a master mix with 2x ddPCR mix and 20x primers, then split the master mix into one that got water (with a ratio of 10ul ddPCR mix, 1ul 20x primer/probe, and 4ul water for every 5ul DNA, to be used at DNA inputs of 1000ng and below) and one that did not get water (with a ratio of 10ul ddPCR mix and 1ul 20x primer for every 9ul DNA, to be used only for inputs of 1800ng).

Droplet Generation: Droplets were generated according to Bio-Rad's instructions. Using a multichannel pipette, 70µl of ddPCR Droplet Generator Oil (Bio-Rad) was slowly pipetted into each oil well of the Droplet Generator DG8 Cartridge (Bio-Rad), and 20µl of sample or control was slowly pipetted into each sample well. The cartridge was covered with a Droplet Generator DG8 Gasket (Bio-Rad) and loaded into the QX100 Droplet Generator (Bio-Rad). After automated droplet generation, the DG8 Gasket was removed and, using a multichannel pipette, each droplet-containing well was slowly aspirated and pipetted into a fresh 96 well, semi-skirted, 250µl PCR plate (Eppendorf).

Heat Sealing: After all samples had been loaded, the plate was covered with a Pierceable Foil Heat Seal (Bio-Rad), loaded into a PX1 PCR Plate Sealer (Bio-Rad), and sealed at 175C for 3s.

PCR: The sealed 96 well plate was loaded onto a 2720 Thermal Cycler (Applied Biosystems). PCR conditions included 95C for 10min, then 45 cycles of 94C for 30s and 59C for 1min, then 98C for 10min, then hold at 4C.

Droplet Reading: Droplets were read on the QX100 Droplet Reader using the ABS program with both Channel 1 (Fam) and Channel 2 (Vic) as unknowns.

Analysis: Gates were set so as to exclude droplets in the negative controls and capture the major droplet-positive population, and were typically midway between the major negative and positive populations. Gates were set to be uniform across all wells. The measured concentration (copies/µl) was multiplied by 20 to get the copies per reaction.

RECIPES:

DNA back extraction buffer (for Trireagent extraction): 4M guanidine thiocyanate (Sigma), 50mM Na citrate (Sigma), and 1M Trizma base (Sigma)

TROUBLESHOOTING:

See Bio-Rad protocols for Digital Droplet PCR

EQUIPMENT:

ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, U.S.A.)

Model B1A gel box, tray, and comb (Owl Separation Systems, Inc, Portsmouth, NH, U.S.A.)

Typhoon FLA 7000 imager (General Electric, Fairfield, CT, U.S.A.)

QX100 Droplet Generator (Bio-Rad, Hercules, CA, U.S.A.)

PX1 PCR Plate Sealer (Bio-Rad, Hercules, CA, U.S.A.)

2720 Thermal Cycler (Applied Biosystems/Life Technologies, Grand Island, NY, U.S.A.)

QX100 Droplet Reader (Bio-Rad, Hercules, CA, U.S.A.)