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HIV-PULSE Wet-lab Protocol V.2

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We use this protocol and it's working

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Abstract

Protocols.io for HIV-PULSE assay, including an updated protocol from the work published in NAR.

Materials

Qubit dsDNA HS Assay Kit (Q32854)
Quant-iT PicoGreen dsDNA Assay Kit (P7589)
CleanPCR beads (CPCR-0050)
Custom size select CleanPCR beads
LongAmp Hot Start Taq DNA Polymerase (M0534L)
dNTPs (Promega, C1141)
PrimeSTAR GXL DNA Polymerase (R050A)
GeneRuler 1 kb Plus DNA Ladder, ready to use (SM1333)
GeneRuler 1 kb DNA Ladder (SM0311)
Native Barcoding Kit 24 V14 kit (ONT, SQK-NBD114-24)
MinION R10.4.1 flow cells (ONT, FLO-MIN114)

Primers (IDT)

	A	B	C	D
	PCR-step	Assay	Name	Sequence (5’ to 3’)
	Pre-amplification	Pinzone outer	Pinzone First PCR Fwd	CCTCAATAAAGCTTGCCTTGAGTGC
			Pinzone First PCR Rev	CCTAGTTAGCCAGAGAGCTCCCAG
		FLIPS/Lee outer	BLOuterF; U5-623F	AAATCTCTAGCAGTGGCGCCCGAACAG
			BLOuterR; U5-601R	TGAGGGATCTCTAGTTACCAGAGTC
	Tagging (PAGE purified)	Pinzone inner	UMI_Pinzone_F2	CAAGCAGAAGACGGCATACGAGATNNNYRNNNYRNNNYRNNNAAGTAGTGTGTGCCCGTCTGTTGTGTGAC
			UMI_Pinzone_R2	AATGATACGGCGACCACCGAGATCNNNYRNNNYRNNNYRNNNGGAAAGTCCCCAGCGGAAAGTCCCTTGTAG
		Lee inner	UMI_Lee_F2	CAAGCAGAAGACGGCATACGAGATNNNYRNNNYRNNNYRNNNGCGCCCGAACAGGGACYTGAAARCGAAAG
			UMI_Lee_R2	AATGATACGGCGACCACCGAGATCNNNYRNNNYRNNNYRNNNGCACTCAAGGCAAGCTTTATTGAGGCTTA
	Amplification (PCR1 to PCR3)	PCR1 to 3	ncec_pcr_fw_v7	CAAGCAGAAGACGGCATACGAGAT
			ncec_pcr_rv_v7	AATGATACGGCGACCACCGAGATC
	Patient barcoding (PCR4)	ID1	ONT_fw1	ACGAGACTGATTCAAGCAGAAGACGGCATACGAGAT

A	B	C	D
		ONT_UD1_R	ACGAGACTGATTAATGATACG GCGACCACCGAGATC
	ID2	ONT_fw2	GCTGTACGGATTCAAGCAGAA GACGGCATACGAGAT
		ONT_UD2_R	GCTGTACGGATTAATGATACG GCGACCACCGAGATC
	ID3	ONT_fw3	ATCACCAGGTGTCAAGCAGAA GACGGCATACGAGAT
		ONT_UD3_R	ATCACCAGGTGTAATGATACG GCGACCACCGAGATC
	ID4	ONT_fw4	TGGTCAACGATACAAGCAGAA GACGGCATACGAGAT
		ONT_UD4_R	TGGTCAACGATAAATGATACG GCGACCACCGAGATC
	ID5	ONT_fw5	ATCGCACAGTAACAAGCAGAA GACGGCATACGAGAT
		ONT_UD5_R	ATCGCACAGTAAAATGATACG GCGACCACCGAGATC
	ID6	ONT_fw6	GTCGTGTAGCCTCAAGCAGAA GACGGCATACGAGAT
		ONT_UD6_R	GTCGTGTAGCCTAATGATACG GCGACCACCGAGATC
	ID7	ONT_fw7	AGCGGAGGTTAGCAAGCAGA AGACGGCATACGAGAT
		ONT_UD7_R	AGCGGAGGTTAGAATGATACG GCGACCACCGAGATC
	ID8	ONT_fw8	ATCCTTTGGTTCCAAGCAGAA GACGGCATACGAGAT
		ONT_UD8_R	ATCCTTTGGTTCAATGATACG GCGACCACCGAGATC

Before start

The HIV-PULSE assay consists of six different PCR steps followed by sequencing on the Minion long read sequencer from Oxford Nanopore Technologies. The main steps are as follows; (i) pre-amplification (to increase sensitivity) and tagging of pre-amplified HIV-1 fragments at each end with a unique UMI, (ii) PCR amplification (split over multiple PCR rounds) will amplify all UMI tagged amplicons making use of the universal synthetic primers attached to each UMI end, (iii) sequencing will be performed on long read sequencer so reads will span the entire HIV-1 fragment with the attached UMI fragments at both ends needed for the bio-informatic workflow (https://github.com/laulambr/longread_umi_hiv).

Pre-amplification: PCR using PrimeSTAR GXL

1 Calculate the x µL volume of sample needed to have an input of 500 ng of genomic DNA.

1.1 More important than the 500 ng is the actual HIV-1 input copies. Minimum 30 copies based on total HIV-1 measurements is advised to obtain at least a single genome.

2 Prepare the master mix according to this principle (if doing replicates, add DNA to master mix, mix well and then divide per sample to ensure 'even' input), beware to adapt the mix according to the required DNA input volume:
a. x µL DNA input
b. 33- x µL NFW

	Stock conc.	Final conc.	Quantity per rx (µL)
DNA			1
5X PrimeSTAR GXL Buffer	5X	1X	10
dNTP Mixture (PS GXL)	2,5 mM	200 µM each	4
Pinzone First PCR F	10 µM	0,2 µM	1
Pinzone First PCR R	10 µM	0,2 µM	1
PrimeSTAR GXL DNA Polymerase	1.25 U/µL	1,25 U / 50 µl	1
Nuclease free water	-	-	32
Total			50

3 Use the following PCR cycling conditions on the cycler. The number of cycles can vary depending on the HIV-1 copy number of the input sample, most important requirement is generating enough yield in order to sequence. Some guidelines:
a. > 2500 total HIV-1 copies/million CD4 T cells: 5 pre-amp cycles
b. < 2500 total HIV-1 copies/million CD4 T cells: 6 pre-amp cycles

A	B	C
STEP	TEMP	TIME
Initial Denaturation	98°C	2 minutes
x Cycles	98°C	10 seconds
	65°C (Pinzone)	15 seconds
	68°C	10 minutes
Final Extension	68°C	10 minutes

	A	B	C
	Hold	4-10°C	

Pre-amplification: cleanup

4 Cleanup of the PCR product using the original CleanPCR beads (CleanNA) at 1.0x ratio. Thus for 50 µL reaction volume of PCR 1, add 50 µL µL of CleanPCR beads.

5 Make sure the CleanPCR magnetic beads are at room temperature.

6 Prepare a fresh 70% Ethanol solution.

7 Vortex the bead solution for 00:00:30 to homogenize the beads. 30s

8 Add 50 µL of bead solution to 50 µL of PCR 1 product and mix by flicking. If you have multiple samples, change tip!

9 Incubate for 00:05:00 at room temperature. 5m

10 Spin down in mini centrifuge.

11 Place the sample on a magnet for 00:02:00 or until the supernatant has cleared. 2m

12 Keep sample on magnet, discard supernatant without disturbing the bead pellet. If you have multiple samples, change tip!

13 Wash the beads with fresh 70% Ethanol by adding 400 µL at the opposite side of the beads (if recipient doesn't hold 400 µL , adapt volume and just make sure that beads are covered in ethanol). Do not resuspend the beads!

14 Incubate for 00:00:30 and remove the ethanol. 30s

15 Repeat the washing steps 13 & 14.

16 Remove excess ethanol by using a P20 pipet (optionally you can also spin down the sample and put back on magnet.

17 Let the beads air dry for 00:00:30 or until the pellet loses its shine (but avoid cracking of DNA since this will make resuspending hard). 30s

18 Remove sample from magnet.

19 Elute the purified DNA by adding 35 µL NFW. Mix by flicking the tube.

20 Incubate for 00:05:00 at room temperature. 5m

21 Put sample on magnet for 00:02:00 or until the supernatant has cleared. 2m

22 Transfer 30 µL of cleaned DNA to new tube.

Tagging: PCR

23 Prepare master mix for PCR2 (can be done during pre-amplification PCR). Use the 30 µL of cleaned DNA from previous step as input.

	Stock conc.	Final conc.	Quantity per rx (µL)
cleaned PCR 1 supernatant			30
5X LongAmp Taq Reaction Buffer	5x	1x	10
dNTP mix	10 mM	100 µM	1.5
Inner UMI HIV PCR Fwd	10 µM	500 nM	2.5
Inner UMI HIV PCR Rev	10 µM	500 nM	2.5
LongAmp Taq DNA Polymerase	5U/µL	0.5U	2
Nuclease free water	-	-	1.5

			50

23.1 Use the inner HIV primers with a UMI tail and not the regular ones. Primers with UMI should be PAGE purified.

24 Use the following PCR cycling conditions on the cycler. 34m

A	B	C
STEP	TEMP	TIME
Initial Denaturation	94°C	1m 15 seconds
2 Cycles	94°C	30 seconds
	58 °C	30 seconds
	65°C	10 minutes
Final Extension	65°C	10 minutes
Hold	4-10°C	

24.1 Do not change the number of cycles since this would mess up the tagging!

Tagging: cleanup

25 Make sure the custom CleanPCR magnetic beads are at room temperature.

25.1 Cleanup of the PCR product using the custom CleanPCR beads at a defined ratio for that custom batch.

26 Prepare a fresh 70% Ethanol solution.

27 Vortex the custom bead solution for 00:00:30 to homogenize the beads. 30s

28 Add 50*ratio µL of bead solution to 50 µL of PCR 2 product and mix by flicking. If you have multiple samples, change tip!

29 Perform clean up as listed in steps 9 to 18.

30 Elute the purified DNA by adding 35 µL Mix by flicking the tube.

31 Incubate for 00:05:00 at room temperature. 5m

32 Put sample on magnet for 00:02:00 or until the supernatant has cleared. 2m

33 Transfer 30 µL of supernatant to new tube.

PCR round 1-2-3: amplification of UMI tagged amplicons

34 Prepare master mix for amplification PCR 1 or PCR 2/3 (Mix is different, for PCR1 30 µL input, while in PCR 2-3 10 µL).
a. For PCR 1: use the 30 µL of cleaned tagged DNA from tagging step as input.
b. For PCR2-3: use the 10 µL of cleaned DNA from previous step as input. Store the remaining 20 µL at -20 °C .

Component	Stock conc.	Final conc.	Quantity (µL)
Eluted UMI tagged DNA			30
5X LongAmp Taq Reaction Buffer	10x	1x	10
dNTP mix	10 mM	200 µM	1.5
Forward_PCR_ONT	10 µM	500 nM	2.5
Reverse_PCR_ONT	10 µM	500 nM	2.5
LongAmp Taq DNA Polymerase	5U/µL	1.25U	2
Nuclease free water	-	-	1.5
Total			50

PCR mix for PCR 1

Component	Stock conc.	Final conc.	Quantity (µL)
Eluted UMI tagged DNA			30
5X LongAmp Taq Reaction Buffer	10x	1x	10
dNTP mix	10 mM	200 µM	1.5
Forward_PCR_ONT	10 µM	500 nM	2.5
Reverse_PCR_ONT	10 µM	500 nM	2.5
LongAmp Taq DNA Polymerase	5U/µL	1.25U	2
Nuclease free water	-	-	1.5
Total			50

PCR mix for PCR 2-3

35 Use the following PCR cycling conditions on the cycler.

A	B	C
STEP	TEMP	TIME
Initial Denaturation	94°C	1m 15 seconds
10 Cycles	94°C	30 seconds
	60 °C	30 seconds
	65°C	10 minutes
Final Extension	65°C	10 minutes
Hold	4-10°C	

PCR round 1-2-3: cleanup

36 Make sure the regular CleanPCR magnetic beads are at room temperature.

37 Prepare a fresh 70% Ethanol solution.

38 Vortex the custom bead solution for 00:00:30 to homogenize the beads. 30s

39 Use the original CleanPCR beads (CleanNA) at 1.0x ratio. Thus for 50 µL reaction volume of PCR product, add 50 µL of CleanPCR beads. Mix by flicking. If you have multiple

samples, change tip!

40 Perform clean up as listed in steps 9 to 18.

41 Elute the purified DNA by adding 35 µL NFW. Mix by flicking the tube.

42 Incubate for 00:05:00 t room temperature. 5m

43 Put sample on magnet for 00:02:00 or until the supernatant has cleared. 2m

44 Transfer 30 µL of supernatant to new tube.

PCR round 4: amplification of UMI tagged amplicons with sample specific primers

45 For the last PCR round, we will tag replicates from the same sample with the same primerset tailed with an index (ID1-8). This will allow later for demultiplexing the replicates per sample based on this index.

Component	Stock conc.	Final conc.	Quantity (µL)
Eluted UMI tagged DNA			20
5X LongAmp Taq Reaction Buffer	10x	1x	10
dNTP mix	10 mM	200 µM	1.5
Forward_PCR_ONT	10 µM	500 nM	2.5
Reverse_PCR_ONT	10 µM	500 nM	2.5
LongAmp Taq DNA Polymerase	5U/µL	1.25U	2
Cresol red	10x	1x	5
Nuclease free water	-	-	6.5
Total			50

PCR mix for PCR 4

46 Prepare master mix for amplification PCR 4. Note that this also uses cresol red and more cleaned material as input to ensure enough yield.

47 Use the following PCR program.

	A	B	C
	STEP	TEMP	TIME
	Initial Denaturation	94°C	1m 15 seconds
	10 Cycles	94°C	30 seconds
		61°C	30 seconds
		65°C	10 minutes
	Final Extension	65°C	10 minutes
	Hold	4-10°C	

47.1 PCR program is slightly adapted from other PCR programs to compensate for longer tailed primers and reduce primer/dimer.

PCR round 4: gel

48 After PCR is finished, transfer 5 µL and visualize on a 1% agarose gel (00:30:00 , 120 Volt) with a GeneRuler 1 kb Plus DNA Ladder (SM1333) for reference. When imaging, use the faint settings. 30m

PCR round 4: cleanup, concentration measurement and pooling

49 Make sure the custom CleanPCR magnetic beads are at room temperature.

49.1 Cleanup of the PCR product using the custom CleanPCR beads at a defined ratio for that custom batch.

50 Prepare a fresh 70% Ethanol solution.

51 Vortex the custom bead solution for 00:00:30 to homogenize the beads. 30s

52 Add 45*ratio µL of bead solution to 45 µL of PCR 4 product and mix by flicking. If you have multiple samples, change tip!

53 Perform clean up as listed in steps 9 to 18.

54 Elute the purified DNA by adding 18 µL NFW. Mix by flicking the tube.

55 Incubate for 00:05:00 at room temperature. 5m

56 Put sample on magnet for 00:02:00 or until the supernatant has cleared. 2m

57 Transfer 15 µL of supernatant to new tube.

58 Quantify with Picogreen, by taking 1 µL of cleaned product as input (performed in duplicate).

59 Based on measured DNA concentrations and estimated overall fragment length (~4-6 kb), calculate for each replicate the equimolar sample pooling strategy.

Sequencing

60 For sequencing protocol, see ONT protocols. Use LFB. Each replicate will be run with a different native barcode from the nanopore barcoding kits (NB01-24). If reusing a flowcell, try to avoid including a barcode already used in previous run.

Extra: make CUSTOM SPRI bead solution 30s

61 Protocol based on 'SPRI size selection protocol for > 1.5-2 kb DNA fragments' from Oxford Nanopore Technologies.

62 Step 1: Prepare the custom buffer by mixing:

Final	Stock	Input (µL)
10 mM Tris-HCl	1 M	20
1 mM EDTA pH 8	0.5 M	4
1.6 M NaCl	5 M	640

11% PEG 8000	50% (w/v)	440
Nuclease free water	-	888
Total		1992

63 Step 2: Transfer bead to Custom buffer

63.1 Bring CleanPCR beads (CleanNA) to room temperature.

63.2 Vortex for 00:00:30 to resuspend beads properly. 30s

63.3 Transfer beads into two 1.5 mL tubes so each contains 1 mL.

63.4 Place the tubes on the magnet, wait until the solution is clear and discard the supernatant.

63.5 Remove the tubes from the magnet, wash with 1 mL of NFW by resuspending the pellet.

63.6 Return the tubes to the magnet, allow beads to pellet and pipette of the supernanant.

63.7 Repeat this NFW wash once more.

63.8 Spin down and place tubes back on magnet. Pipette off any residual water.

63.9 Pool the two bead pellets together by resuspending them in 200 µL of Custom buffer.

63.10 Transfer the beads into the remaining Custom buffer.

64 Step 3: Test different ratios of new batch of Custom buffer on DNA ladder to determine perfect ratio.

We generally test a range of different ratios including 0.8x, 0.9x, 1.0x, 1.1x and 1.2x conditions.

new 0.8
new 0.9
new 1.0
new 1.1
new 1.2

64.1 For each ratio to be tested, take 3 µL of GeneRuler 1 kb DNA Ladder (SM0311), add to 17 µL of TE.

64.2 Add to this the required volume of custom buffer (for 1.0x add for instance 20 µL).

64.3 Incubate for 00:05:00 at room temperature. 5m

64.4 Put on magnet for two minutes or until supernatant has cleared.

64.5 Discard supernatant.

64.6 Wash by adding 200 µL 70% ethanol, leave 00:00:30 and remove. 30s

64.7 Repeat ethanol step once more.

64.8 Remove residual ethanol.

64.9 Add 30 µL NFW buffer.

64.10 Take from magnet and resuspend, incubate for 00:05:00 5m

64.11 Put on magnet for 00:02:00 until supernatant has cleared. 2m

64.12 Remove 30 µL cleaned ladder and put on visualize on a 1% agarose gel.