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Version 2

SIMPLseq protocol V.2

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SIMPLseq



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We use this protocol and it's working

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Disclaimer

Always remember hood sterilization before use

Abstract

SIMPLseq is a 6-locus *Plasmodium falciparum* AmpSeq miniplex optimized for high-sensitivity analyses that also integrates a contamination detection system based on well-specific inline barcodes applied during PCR1.

The 'six informative malaria parasite loci' contained within the panel represent the following 6 genes:

- CSP (Pf3D7_0304600)
- TRAP (Pf3D7_1335900)
- SERA8 (Pf3D7_0207300)
- SURFIN 4.2 (Pf3D7_0424400)
- WDCP (Pf3D7_1410300)
- KELT (Pf3D7_1475900)

Attachments



[barcodes.xlsx](#)

9KB

Image Attribution

Figure 1. PCR1 product Bioanalyzer traces before (left) and after (right) ExoSap digestion.

Figure 2. Bioanalyzer traces for PCR2 product pools after bead clean-up. 4CAST libraries (example shown at right) often require more elaborate, aggressive clean-up steps.

Guidelines

(FYIs)

Step 1: The 5' sequences TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG and GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG represent Illumina adapters associated with Nextera library preparation kits. These serve as common binding sites for PCR2 primers as well as for standard sequencing primers during Illumina sequencing (SBS).

Step 2: This implies a total primer mix concentration of 60 μM , which is in the upper range of typical PCR primer concentrations. Sometimes sensitivity can be increased by reducing primer concentrations (less primers $\rightarrow$ less potential for off-target binding), but this was not found to be the case for the SIMPLseq assay.

Step 3: We recommend increasing template volume input when parasitemias are expected to be low. This is simply because, at low parasitemias, small aliquots (3 μl) of extracted DNA may not contain any parasites at all. For example, let us consider a parasitemia level of ~ 1 p/ μl in whole human blood. This likely becomes <0.2 p/ μl through the course of DNA extraction. A random 4 μl extraction aliquot is expected to contain less than a single parasite on average if distribution is homogeneous; increasing the PCR1 input volume helps increase our chances of capturing at least one parasite genome.

Step 6: 29x cycles were chosen as a suitable balance between increasing PCR yield and limiting reaction artifacts. Another significant 'knob' to turn in PCR protocols is annealing temperature; at 57°C , SIMPLseq uses a relatively permissive annealing temperature and thus requires caution with $>30\text{x}$ PCR1 cycle counts.

Steps 7-11: The ExoSap step digests/removes small fragments (predominantly <75 bp), especially primers, from PCR1 products, as visualized in the protocol. In our hands, this leads to clearer PCR2 product signal (Bioanalyzer traces). This step does not generate significant additional cost (ca. 25-50 cents per sample) but implies further plate handling (e.g., sealing/unsealing, during which contamination is always a risk) and adds another ca. 45 minutes to the protocol. The subsequent dilution step helps minimize residual components (e.g., glycerol, EDTA) but a similar PCR1 product dilution would also take place in the absence of this digestion step.

Step 14: The phosphorothioate bond within the indexing primers represents a phosphate modification that protects the 3' end of the primer from polymerase exonuclease activity, ensuring accurate integration of index sequences during PCR2.

Step 19: Performing an additional clean-up, (i.e, using the output of 0.8x clean-up as input for another 0.8x clean-up) is more likely required when libraries include many very low (<1 p/ μl) or negative samples. If you suspect this to be the case, make sure to plan out your clean-ups with respect to required input/output volumes and concentration (note that we need 2-5 nM (preferably 5 nM, see step 30) for Illumina's final 'Denature and dilute' procedure). It is not uncommon to lose $>50\%$ of the target range concentration, due to factors including incomplete initial bead binding, losses during pellet washing, excessive pellet drying, or incomplete solubilization into the elution buffer (heating helps!). Further bead cleanups may be done if primer dimers remain.

Step 28: The inclusion of additional samples (e.g., the current PhiX aliquot, libraries and PhiX from previous runs, multiple Standard 0's) is very helpful to help accurately triangulate concentration, as well as using corresponding Qubit and Bioanalyzer information. Ideally, users also keep a long-term log of quantitation results and associated sequencing performance (especially clustering density) given that one-time qPCR readings are susceptible to misinterpretation.

Steps 30-31: Please note that, because we start Illumina's 'Denature and dilute' guide with 5 nM library instead of the standard 4 nM, final output concentration becomes ca. 17.5 pM when following the guide's directions for, e.g., 14 pM.

Materials

- SIMPLseq PCR1 primers (sequences indicated in protocol) – purchase from IDT
- KAPA HiFi HotStart ReadyMix (2x) – Roche 07958927001
- Exonuclease I – Thermo Scientific EN0581
- FastAP – Thermo Scientific EF0651
- Nextera UD index sequences [10 µM plate] – purchase from IDT
- AMPure XP beads – Beckman-Coulter A63880
- 80% ethanol – via Sigma-Aldrich E7023 stock or similar
- Magnetic rack for 0.2 ml tubes – EpiCypher 10-0008 or similar
- 10 mM Tris-Cl pH 8.5 – Teknova T1275 or similar
- Qubit dsDNA Quantification Kit – Thermo Scientific Q32851
- Bioanalyzer High Sensitivity DNA Reagents – Agilent 5067-4627
- KAPA Library Quantification Kit – Roche KK4824
- MiSeq Reagent Kit v2 (500-cycles) – Illumina MS-102-2003

Other items are standard to most molecular laboratories (thermocyclers, plate spinners, centrifuge, Qubit, Bioanalyzer, etc. – see protocol).

SIMPLseq primer cocktail preparation (for a 96-well plate)

- 1 Combine 10 µl of each PCR1 primer at 100 µM into an 1.5 ml Eppendorf tube. Store at -20°C.

PCR1 primer sequences (5'-3'):

- CSP_Fw:
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTAAGGAACAAGAAGGATAATACCA
- CSP_Rv:
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAATGACCCAAACCGAAATG
- TRAP_Fw:
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCCAGCACATGCGAGTAAAG
- TRAP_Rv:
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAACCCGAAAATAAGCACGA
- SERA8_Fw:
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACGAATTGTATTTCTCCTAATATGTGC
A
- SERA8_Rv:
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGGCACATAATCCAATAGATGTCCC
- SURFIN_Fw:
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGCCAAAAAGGAGATCAGCAAAG
- SURFIN_Rv:
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGCTTCATCCAAACATACATCCTC
- WDCP_Fw:
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGTCTACACGCGTATTTCATAGCT
- WDCP_Rv:
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGACGAAAAAATTGGGAACATACCA
- KELT_Fw:
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*****AGAAAATAACAAGAGAATCAG
GGCAA
- KELT_Rv:
TCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*****TCCATCTCTAGGTAATAACGT
ACCAT

The asterisks (*****) indicate the optional use of inline-barcoded primer pairs. To maintain assay sensitivity, we only apply this feature to a single primer pair (KELT). We use a combinatorial arrangement of barcode sequences for each 96-well plate (see layout example in barcodes.xlsx).

- 2 Perform a 60/40 dilution (e.g., 120 µl primer mix + 80 µl nuclease-free water (NF dH₂O) to achieve 5 µM per primer working concentration.

PCR1

- 3 Prepare PCR1 master mix using the following *minimum volumes per sample:

*Increase DNA and all other inputs indicated in steps 3-5 by a factor of 3 if you can.

- 5 µl KAPA HiFi HotStart ReadyMix (2x) – Roche 07958927001
- 1.5 µl PCR1 primer mix [5 µM per primer]
- 4 Add 6.5 µl PCR1 master mix to every plate well.
- 5 Add 4 µl sample genomic DNA (or control templates, placed randomly throughout the plate).
- 6 Place plate on a thermocycler with the following amplification settings:

- 1× 95 °C – 3:00
- 29× 98 °C – 00:20 57 °C – 00:15 72 °C – 00:30
- 1× 72 °C – 01:00
- 1× 4 °C – hold

ExoSap digestion

- 7 Transfer 5 µl PCR1 product into new plate.
- 8 Using using Thermo Scientific EN0581 and EF0651, make digestion master mix on ice: 0.25 µl Exonuclease I (5 u) + 1.0 µl FastAP (1 u) per sample. Make enough volume for twice your number of samples given the viscosity of the mix.
- 9 Add 1.25 µl digestion master mix to your plate of 5 µl PCR1 samples.
- 10 Incubate for 30 minutes in thermocycler: 37 °C – 15:00, 85 °C – 15:00, 4 °C – hold.
- 11 Add 12.5 µl NF dH₂O to each sample (2-to-1 dilution) and mix thoroughly.

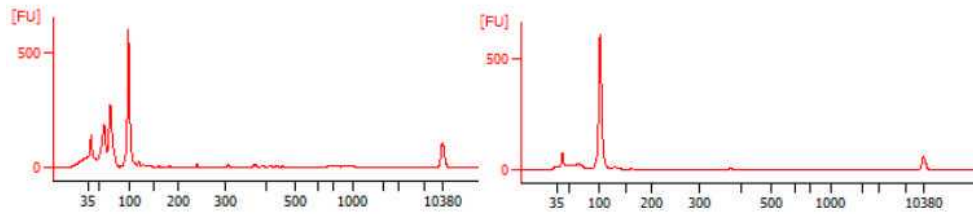


Figure 1. PCR1 product Bioanalyzer traces before (left) and after (right) ExoSap digestion.

PCR2

12 Prepare PCR2 master mix using the following volumes per sample:

- 5 µl KAPA HiFi HotStart ReadyMix (2x) – Roche 07958927001
- 2 µl NF dH₂O

13 Add 7 µl PCR2 master mix to every plate well.

14 Add 2.2 µl Nextera unique dual index [10 µM plate] into each well.

These index sequence pairs are of the form:

5' – AATGATACGGCGACCACCGAGATCTACAC – 10 bp index – TCGTCGGCAGCGT*C – 3'

5' – CAAGCAGAAGACGGCATACGAGAT – 10 bp index – GTCTCGTGGGCTCG*G – 3'

The asterisk (*) indicates phosphorothioate bond.

These can be purchased with standard de-salt purification in ca. 10 µl volumes per well.

15 Add 3 µl digested/diluted PCR1 product to each well.

16 Place plate on a thermocycler with the following amplification settings:

- 1× 95 °C – 03:00
- 10× 98 °C – 00:20 65 °C – 00:30 72 °C – 00:30
- 1× 72 °C – 01:00
- 1× 4 °C – hold

Sample pooling and clean-up (removal of fragments except the ca. 300-550 bp target range)

- 17 Combine equal volumes (~4 µl) of each sample into a 2.0 ml Eppendorf tube. If there are many samples, use a multichannel and combine into a reagent reservoir, mix well, and then add into the tube.
- 18 Perform clean-up using AMPure XP beads (Beckman-Coulter A63880):
 - a) Transfer 100 µl of combined sample into a PCR tube and add 80 µl beads (0.8x clean-up). Mix well.
 - b) Incubate at room temperature for 5 minutes.
 - c) Place on magnetic rack for 5 minutes.
 - d) Discard supernatant and wash twice with 200 µl of fresh 80% ethanol while still on the rack. Incubate for 30 seconds between the two washes.
 - e) Remove remaining traces of ethanol via pipette and allow to evaporate for 2 minutes.
 - f) Elute in 50 µl 10 mM Tris-HCl pH 8.5, close caps and incubate for 8-10 minutes at 57 °C, then place on magnetic rack and allow the pellet to form (~5 min).
 - g) Transfer 50 µl supernatant to new tube. Avoid residual beads, e.g., aspirate slightly under the total volume (49 µl) if clean transfer is otherwise not possible.
- 19 If necessary, repeat 0.8x clean-up for 50 µl library (i.e., use 40 µl beads). Elute in ~15 µl at step g.

Bioanalyzer QC / library selection

- 20 Fragment size-specific DNA quantification can be performed using Bioanalyzer High Sensitivity DNA Reagents (Agilent 5067-4627). It is best to first verify via Qubit dsDNA Quantification Kit (Thermo Scientific Q32851) that your input concentrations fall within Bioanalyzer's linear dynamic range (ca. 50 pg/µl – 10 ng/µl). In our experience, size-selected libraries (created in step 19) generally require ca. 1:100 dilution (in 10 mM Tris-Cl pH 8.5 + 0.1% Tween-20) to reach appropriate concentrations for Bioanalyzer.
- 21 Follow manufacturer's instructions to run the Bioanalyzer High Sensitivity DNA protocol. Expected fragment size are 473 bp (CSP), 500 bp (TRAP), 426 bp (SERA8), 415 bp (SURFIN), 425 bp (WDGP), and 381 bp (KELT). See results below for double 0.8x clean-up on a low-parasitemia sample set (middle plot) in comparison to what you might get with the 4CAST assay (right plot).

22

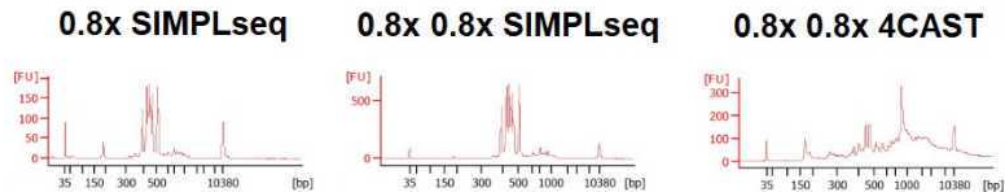


Figure 2. Bioanalyzer traces for PCR2 product pools after bead clean-up. 4CAST libraries (example shown at right) often require more elaborate, aggressive clean-up steps.

qPCR quantification of selected library

- 23 Thaw the qPCR reagents (KAPA Library Quantification Kit – Roche KK4824), except the SYBR FAST component, which will be thawed just before use. Gently vortex and centrifuge the kit controls before use.
- 24 Dilute sample library such that it enters the 0.1-10 pM range (this represents the upper-middle range of the kit's standard curve – see next step). For example, for a library preliminarily estimated as 20 nM via Bioanalyzer or Qubit, dilute 1:10,000 using NF dH₂O. This yields 2pM.
- 25 Dilute Standard 0 (200 pM stock concentration) 1:100.

Standards (S1-S6) do not require dilution.

Standard 1 – 20 pM

Standard 2 – 2 pM

Standard 3 – 0.2 pM

Standard 4 – 0.02 pM

Standard 5 – 0.002 pM

Standard 6 – 0.0002 pM

Standard 0 – 2 pM after 1:100 dilution

- 26 Prepare qPCR master mix (Roche KK4824) using the following volumes per sample:

- 10 µl KAPA SYBR FAST qPCR Master Mix (2X)
- 2 µl Primer PreMix
- 4 µl NF dH₂O

- 27 Add 16 µl of qPCR master mix to each well.

- 28 Plate the standards in triplicate, 4 µl per well. Then plate 4 µl sample library and negative control (NF dH₂O), likewise in triplicate.



It is optional but recommended to also add any available reference libraries (e.g., libraries which clustered well in prior sequencing runs, and the PhiX aliquot to be used in the current run). This helps confirm accurate quantitation. 

29 Seal and spin for ~30 seconds before placing in light cycler with the following amplification settings:

- 1× 95 °C – 5:00
- 35× 95 °C – 00:30 60 °C – 00:45 *with data acquisition

Final sequencing preparation

30 Dilute library to 5 nM for Illumina MiSeq.

31 Follow Illumina's guide to *Denature and Dilute Libraries for the MiSeq System*, targeting a final library concentration of 14 pM with 20% PhiX.