Jul 15, 2025 Version 2

PfSMARRTer- *Plasmodium falciparum* Streamlined Multiplex Antimalarial Resistance and Relatedness Testing (PfSMARRT v1.13) V.2

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Protocol status: Working
We use this protocol and it's working

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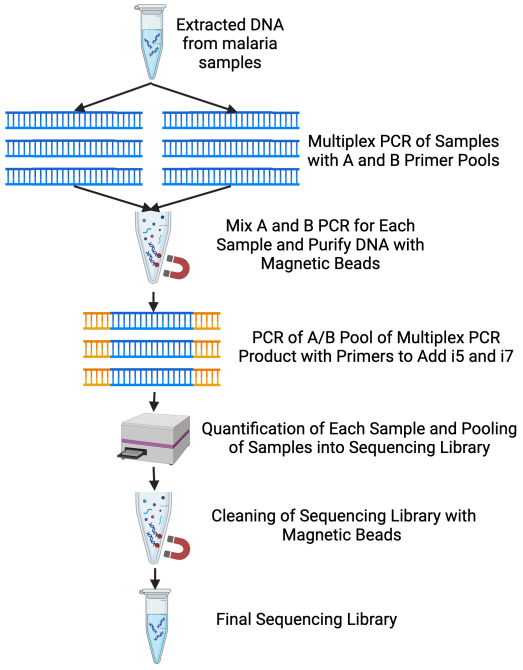
Abstract

PfSMARRTer- An updated version of PfSMARRT

This is a multiplex PCR protocol for amplifying ama1, crt, mdr1, dhfr, dhps, and k13. The PCR primers were adopted from:

- AMA1 - **PMID:29246158**
- CRT - designed for this protocol
- MDR1 - **PMID:15132750** (MDR184 designed for this protocol)
- DHFR & DHPS - **PMID:15273102** and Am J Trop Med Hyg. 2008 Jun; 78(6): 892–894
- K13 – designed for this protocol
- HeOME: Aranda-Díaz A, Vickers EN, Murie K, Palmer B, Hathaway N, Gerlovina I, et al. Sensitive and modular amplicon sequencing of diversity and resistance for research and public health. bioRxiv. 2024.



Schematic of Pf-SMARRTer Protocol

Image Attribution

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Guidelines

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www.med.unc.edu/infdis/ideel

	A	B	C	D
	Version	**Date**	**Authors**	**Notes**
	1.1	18-Mar-2022	K. Wamae	- draft protocol, primer pooling and PCR optimisation
	1.2	10-Jun-2022	K. Wamae	- adjusted primer pool ratios
	1.3	1-Jul-2022	K. Wamae	- adjusted primer pool ratios - replaced K13-B primer (F2 for F)
	1.4	2-Sept-2022	K. Wamae	- renamed *k13* primers across all document versions to indicate loci covered - changed the primer-pool ratios in ***section 1.2*** back to those indicated in ***protocol version 1.2*** since they performed better
	1.5	19-December	K.Wamae	- Included expected amplicon lengths based on the 3D7 lab isolate
	1.6	11-January 23	J. Juliano	-Include new primers for mdr184, crt, k13 622I and HeOME - Add normalisation of P1 and P2 products into library prep
	1.7	27-March 23	J. Juliano J. Bailey	-Rebalancing of primers to try to normalise products - Updated primer location and size table
	1.8	27-April 23	J. Juliano J. Bailey	- Rebalance of primers
	1.9	14-June 23	J. Juliano	- Rebalance of primers
	1.10	04-Nov-23	J. Sadler J. Juliano	- Standardization of amplicon pooling - Removal of quantification before amplicon pooling
	1.11	12-Feb-24	J. Juliano J. Sadler	- Conversion to Nextera Prep - Addition of dhfr 164 and pfmdr1 1246 - Removal of pfmdr1 1034

	A	B	C	D
	1.12	05-14-24	J. Juliano J. Sadler C. Henelley	- Increased PfCRT from 10uL fwd and rev primer → 15uL
	1.13	07-02-24	J. Juliano J. Sadler A. Simkin	-K13-F forward changed to address lack of coverage -K13-G forward changed to address lack of coverage

Materials

1.1 List of primers.

	A	B	C
	Gene	Primer Name	Primer Sequence (5'-3')
	ama1	AMA1_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCATCAGGGAAATGTCCAGT
		AMA1_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTTCCTGCATGTCTTGAACA
	crt	PFCRT_F_v2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGTGGAGGTTCTTGTCTTGG
		PFCRT_R_v2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGTTGTGAGTTTCGGATGTTACA
	mdr1	MDR1_86_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGTATGTGCTGTATTATCAGGAGGAAC
		MDR1_86_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAATTGTACTAAACCTATAGATACTAATGATAATATTATAGG
		MDR1_184_F_v2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGAGTTCAGGAATTGGTACGA
		MDR1_184_R_v2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCCTCTTCTATAATGGACATGGT
		MDR1_1246_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAAAATGTAAATGAATTTTCAAACCAATCTGGA
		MDR1_1246_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATAGCAGCAAACTTACTAACACGTTTAA
	dhfr	DHFR_51_59_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGAGGTTTTTAATAACTACACATTTAGAGGTCT
		DHFR_51_59_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTATCATTTACATTATCCACAGTTTCTTTGTT
		DHFR_108/164_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAAAATTACAAAATGTTGTAGTTATGGGAAGAA
		DHFR_108/164_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCTAAAAATTCTTGATAAACAACGGAACCT
	dhps	DHPS_437_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGAAATGATAAATGAAGGTGCTAGTGT
		DHPS_437_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAATACAGGTACTACTAAATCTCTTTCACTAATTTTT
		DHPS_540_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAATGCATAAAAGAGGAAATCCACAT

	A	B	C
		DHPS_540_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCGCAAATCCTAATCCAATATCAA
		DHPS_581_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTCGTTATAGGATACTATTTGATATTGGAT
		DHPS_581_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGGCAATAAATCTTTTTCTTGAATA
		DHPS_613_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGGATTAGGATTTGCGAAGAAAC
		DHPS_613_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTTGTGTATTTATTACAACATTTTGATCATTC
	k13	K13-A-432-466_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAAAGTGAAGCCTTGTTGAAAGAAG
		K13-A-432-466_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTACACATACGCCAGCATTGTTG
		K13-B-461-531_F2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGATGGTGTAGAATATTTAAATTCGATG
		K13-B-461-531_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTACCATTTGACGTAACACCACA
		K13-C-513-570_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTTGAAACTGAGGTGTATGATCG
		K13-C-513-570_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTGATGATCTAGGGGTATTCAA
		K13-F-567-630_F2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCGAATGTAGAAGCATATGATCA
		K13-F-567-630_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGGTAATTAAAAGCTGCTCCTGA
		K13-G-660-709_F2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAAGCTAGAAGTTCAGGAGC
		K13-G-660-709_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCCAAGCTGCCATTCATTTG
	HeOME	HeOME-A F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTTTAGTTTCGGTATTTTGTGTTCCTCTT
		HeOME-A R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAGAAATTTATCAGAGTTACAAAAGGGAAATC
		HeOME-B F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTTATCCTTATCATTATTTCCATCATTTTCTGG
		HeOME-B R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAAAATAAAAGGAACAATGTAATGGTTGAAAA

	A	B	C
		HeOME-C F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCCCGAAAACATATCACTAGATCCAT
		HeOME-C R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAAAATTAAACATGATGCCACATTTTAGTAGT
		HeOME-D F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGCTATCATTACATGCTGACACAATAT
		HeOME-D R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGATTAGTTGTGGAGATGATAAAACTATCAAATT
		HeOME-E F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAATTTTCTTATATAACCTAAGTTGATGACTTGG
		HeOME-E R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGAACAGATGAAGTAACTACTCGATTAAATGA
		HeOME-F F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATCTTTTTCGTTGTATGTGCATAATCA
		HeOME-F R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAACATAATTCTAATGATATTGACCTTGTGCA
		HeOME-G F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACATTCACACAAATAGAAAAATCTTCATTTTTC
		HeOME-G R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATCAATAATCAAAATCATGATAACAACCAATTT
		HeOME-H F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAATAACTTAAATAAAAATATGGACGGCTCC
		HeOME-H R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACATTCTTTCAATGCTTCCGAAA

1.2 Primer pool ratios

- Mix the primers above (both forward and reverse) into pools 1 and 2 in the volumes below.

	A	B	C	D
	Primer	**Pool 1 volume**	**Primer**	**Pool 2 volume**
	AMA1	20	PFCRT_v2	18
	MDR1-86	20	MDR1-184_v2	12

	A	B	C	D
	MDR1-1246	20	K13-B	18
	K13-A	20	K13-F	12
	K13-C	25	DHPS-540	24
	K13-G	15	DHPS-613	18
	DHPS-436-437	15	DHFR-51-59	24
	DHPS-581	15	HeOME-A	24
	DHFR-108/164	25	HeOME-C	18
	HeOME-B	25	HeOME-E	18
	HeOME-D	20	HeOME-G	36
	HeOME-F	15	HeOME-H	12

Note

The volume listed for each target should be added for both forward and reverse primers. For example, AMA1 has a volume of 20µL listed. Add 20µL of both forward and reverse primer to the pool, 40µL total for the target if making one batch.

Table 1.3 - I7 index

	A	B	C	D
	Beginning	Index	End	To order
	CAAGCAGAAGACGGCATACGAGAT	CGCTCAGTTC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATCGCTCAGTTCGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TATCTGACCT	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTATCTGACCTGTCTCGTGGGCTCGG

	A	B	C	D
	CAAGCAGAAGACGGCATACGAGAT	TCGGATGTCG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTCGGATGTCGGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	CTTATGGAAT	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATCTTATGGAATGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TCCTATTGTG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTCCTATTGTGGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	GCGCGATGTT	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATGCGCGATGTTGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	AGAGCACTAG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATAGAGCACTAGGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TGCCTTGATC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTGCCTTGATCGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	CTACTCAGTC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATCTACTCAGTCGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TCGTCTGACT	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTCGTCTGACTGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	GAACATACGG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATGAACATACGGGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	CCTATGACTC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATCCTATGACTCGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TAATGGCAAG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTAATGGCAAGGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	GTGCCGCTTC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATGTGCCGCTTCGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	CGGCAATGGA	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATCGGCAATGGAGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	GCCGTAACCG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATGCCGTAACCGGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	AACCATTCTC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATAACCATTCTCGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	GGTTGCCTCT	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATGGTTGCCTCTGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	CTAATGATGG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATCTAATGATGGGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TCGGCCTATC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTCGGCCTATCGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	AGTCAACCAT	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATAGTCAACCATGTCTCGTGGGCTCGG

	A	B	C	D
	CAAGCAGAAGACGGCATACGAGAT	GAGCGCAATA	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATGAGCGCAATAGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	AACAAGGCGT	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATAACAAGGCGTGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	GTATGTAGAA	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATGTATGTAGAAGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TTCTATGGTT	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTTCTATGGTTGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	CCTCGCAACC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATCCTCGCAACCGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TGGATGCTTA	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTGGATGCTTAGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	ATGTCGTGGT	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATATGTCGTGGTGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	AGAGTGCGGC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATAGAGTGCGGCGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TGCCTGGTGG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTGCCTGGTGGGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TGCGTGTCAC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTGCGTGTCACGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	CATACACTGT	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATCATACACTGTGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	CGTATAATCA	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATCGTATAATCAGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TACGCGGCTG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTACGCGGCTGGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	GCGAGTTACC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATGCGAGTTACCGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TACGGCCGGT	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTACGGCCGGTGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	GTCGATTACA	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATGTCGATTACAGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	CTGTCTGCAC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATCTGTCTGCACGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	CAGCCGATTG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATCAGCCGATTGGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TGACTACATA	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTGACTACATAGTCTCGTGGGCTCGG

	A	B	C	D
	CAAGCAGAAGACGGCATACGAGAT	ATTGCCGAGT	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATATTGCCGAGTGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	GCCATTAGAC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATGCCATTAGACGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	GGCGAGATGG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATGGCGAGATGGGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TGGCTCGCAG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTGGCTCGCAGGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TAGAATAACG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTAGAATAACGGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TCCATGTTGC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTCCATGTTGCGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TATCCAGGAC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTATCCAGGACGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	AGTGCCACTG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATAGTGCCACTGGTCTCGTGGGCTCGG

Table 1.4 - I5 index

	A	B	C	D
	Beginning	Index	End	To order
	AATGATACGGCGACCACCGAGATCTACAC	TCGTGGAGCG	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACTCGTGGAGCGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	CTACAAGATA	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACCTACAAGATATCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	TACGTTCATT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACTACGTTCATTTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	TGCCTGGTGG	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACTGCCTGGTGGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	TCCATCCGAG	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACTCCATCCGAGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	GTCCACTTGT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACGTCCACTTGTTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	TGGAACAGTA	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACTGGAACAGTATCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	CCTTGTTAAT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACCCTTGTTAATTCGTCGGCAGCGTC

	A	B	C	D
	AATGATACGGCGACCACCGAGATCTACAC	GTTGATAGTG	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACGTTGATAGTGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	ACCAGCGACA	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACACCAGCGACATCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	CATACACTGT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACCATACACTGTTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	GTGTGGCGCT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACGTGTGGCGCTTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	ATCACGAAGG	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACATCACGAAGGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	CGGCTCTACT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACCGGCTCTACTTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	GAATGCACGA	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACGAATGCACGATCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	AAGACTATAG	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACAAGACTATAGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	TCGGCAGCAA	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACTCGGCAGCAATCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	CTAATGATGG	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACCTAATGATGGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	GGTTGCCTCT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACGGTTGCCTCTTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	CGCACATGGC	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACCGCACATGGCTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	GGCCTGTCCT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACGGCCTGTCCTTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	CTGTGTTAGG	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACCTGTGTTAGGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	TAAGGAACGT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACTAAGGAACGTTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	CTAACTGTAA	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACCTAACTGTAATCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	GGCGAGATGG	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACGGCGAGATGGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	AATAGAGCAA	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACAATAGAGCAATCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	TCAATCCATT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACTCAATCCATTTCGTCGGCAGCGTC

	A	B	C	D
	AATGATACGGCGACCACCG AGATCTACAC	TCGTATG CGG	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACTCGTA TGCGGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	TCCGAC CTCG	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACTCCG ACCTCGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	CTTATGG AAT	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACCTTAT GGAATTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	GCTTAC GGAC	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACGCTTA CGGACTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	GAACATA CGG	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACGAAC ATACGGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	GTCGATT ACA	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACGTCG ATTACATCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	ACTAGC CGTG	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACACTAG CCGTGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	AAGTTG GTGA	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACAAGTT GGTGATCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	TGGCAA TATT	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACTGGC AATATTTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	GATCAC CGCG	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACGATCA CCGCGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	TACCATC CGT	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACTACCA TCCGTTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	GCTGTA GGAA	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACGCTG TAGGAATCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	CGCACT AATG	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACCGCA CTAATGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	GACAAC TGAA	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACGACA ACTGAATCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	AGTGGT CAGG	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACAGTG GTCAGGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	CCATCT CGCC	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACCCATC TCGCCTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	CTGCGA GCCA	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACCTGC GAGCCATCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	CGTTATT CTA	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACCGTTA TTCTATCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	GCAACA TGGA	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACGCAA CATGGATCGTCGGCAGCGTC

	A	B	C	D
	AATGATACGGCGACCACCGAGATCTACAC	GTCCTGGATA	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACGTCCTGGATATCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	CAGTGGCACT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACCAGTGGCACTTCGTCGGCAGCGTC

Reagents

- 80% Ethanol
- Elution Buffer
- Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #**KK2601
- Molecular Grade Water
- Pre-Combined UDI 96-well plate at 10µM total concentration (5µM each index, 10µM total concentration)
- 2X QIAGEN Multiplex PCR Master Mix **Qiagen Catalog #**206143 or QIAGEN Multiplex PCR Kit **Qiagen Catalog #**206145
- SpeedBead Mix (PMCID: **PMC6791352**)
- Quant-iT dsDNA Pico Green assay kit (Invitrogen) **Life Technologies Catalog #**P7589
- Invitrogen™ Qubit™ dsDNA Quantification Assay Kits **Fisher Scientific Catalog #**Q32850
- Optional: If needed, fragment analyzer supplies (such as https://www.agilent.com/en/product/automated-electrophoresis/tapestation-systems/tapestation-dna-screentape-reagents/dna-screentape-analysis-228260)
- Optional: Commercial Illumina Library Quantification Kit with standards

Consumables

- Nuclease-free PCR microtubes/strips/plates (preferably LoBind).
- Nuclease-free 1.5µL Eppendorf tubes (for master mixes, preferably LoBind).
- Freezer blocks for microtubes and 1.5µL tubes to hold plates and tubes during processing.
- P200 pipettor and tips
- P20 pipettor and tips
- 25µL reagent reservoir (3)
- 96-well Flat-bottomed Assay Plates (Black)

Experiment set up

- Thaw reagents and sample DNA on ice.
- Clean pipettes and working bench tops with 70% ethanol.
- UV-treat the working surface, all equipment, and tubes/strips.
- Briefly vortex and centrifuge each reagent before use.

Methods

1 Dilute the [M] 100 micromolar (µM) stock primers down to [M] 10 micromolar (µM) working solutions for each forward and reverse primer.

2 Prepare the two primer pools as indicated in section **1.1 [In materials]** by mixing all forward and reverse primers in the ratios shown in section **1.2 [In materials]**.

3 Prepare the PCR master mix based on the table below.

- Since there are two primer pools, the recipe below applies to both pools 1 and 2.
- If samples are going to be sequenced in duplicate, this translates to translates to four PCR reactions per sample, i.e., two PCR replicates for primer pool 1 and two PCR replicates for primer pool 2.
- Calculate the amount of reagents needed with a 10% excess to account for pipette loss.

	A	B
	Reagents for a 25µl reaction	**1x Reaction**
	Qiagen PCR Master Mix	12.5µL
	Primer Pool+	2.5µL
	Template DNA*	2.0-5.0µL
	PCR grade water^	5.0-8.0µL

+The “Primer Pool” represents one of two pools in separate reactions.
*Template DNA volume can be adjusted depending on the concentration of DNA by changing the amount of PCR grade water in the reaction.
^ Amount of water varies inversely with Template DNA volume for a total of 10ul

3.1 All reagents, PCR master mix, primer pools, and samples should be held in freezer blocks (tube or 96-well) unless stated otherwise.

4 Ensure that PCR reactions are labelled to be able to identify the sample therein and which primer pool (A or B) is being used in the PCR.

4.1 All assays are carried out with positive and negative controls (preferably 2 of each). For a positive control, we use a mocked dried blood spot containing an estimated parasite density of 100 Pf/µL. The positive control you use should be extracted the same way as samples to be tested. For non-template control, we recommend using human DNA at a clinically relevant concentration (e.g. 16.77 ng/). If human DNA is not available, water can be used as a non-template control.

4.2 Controls should be run through the entire library prep process and checked in Step 30.

5 Combine the master mix and sample DNA, seal caps/strips/plates, and place the reaction in a thermal cycler using the following conditions:

	A	B	C	D
	Step	**Duration**	**Cycles**	**Temperature**
	Initial activation step	15 min	1	95° C
	Denaturation	***30 sec***	45	***94° C***
	Annealing	***90 sec***		***60° C***
	Extension	***90 sec***		***72° C***
	Final Extension	10 min	1	72° C
	Hold	Indefinite	1	4° C

Mixing of PCR products & 1:1 Speed-Bead purification

48m

6 Bring Speed-Beads to Room temperature (00:30:00). 30m

7 In a 96-well LoBind PCR plate, combine the entire P1 and P2 PCR reaction volume into a single well creating the final multiplex amplicon pool.

- Mix fresh 80% Ethanol using 200 proof ethanol (100%, anhydrous) and molecular grade water.

8 Vortex Speed-Beads thoroughly then add 50 µL to each well. Mix gently by pipetting.

9 Allow the mixture to incubate at Room temperature for 00:10:00 . 10m

9.1 If there are droplets on the sides of the wells following incubation, cover the plate with a foil seal and briefly spin the plate to avoid losing product.

10 Place the plate on a 96-well magnetic block and wait 00:02:00 - 00:03:00 for the solution to clear. 3m

11 Aspirate the clear supernatant and discard.

12 Dispense 120 µL 80% Ethanol into each well, incubate 00:00:30 , then aspirate to wash. 30s

12.1 Repeat for a total of 2 washes.

13 Aspirate final drops of Ethanol with P20 pipette tips. Allow bead pellets to dry momentarily 00:00:30 - 00:01:00 . 1m 30s

14 Remove plate from the magnet.

15 Dispense 25 µL of Elution Buffer onto each bead pellet. Mix by pipetting to resuspend DNA.

16 Cover plate with foil seal and briefly centrifuge to collect product in the bottom of wells before storing or proceeding to library preparation. *The beads can remain in the eluant and may be added to the library preparation reaction. The eluant is not transferred to a new plate to limit cross-contamination of concentrated amplicon products.*

Spot-quantitation

17 Using Qubit dsDNA HS reagent according to manufacturer instructions, spot-quantify three samples to ensure amplified DNA was not lost during purification.

18 If needed, run the purified products on a fragment analyzer (e.g. TapeStation) to ensure that you have the desired PCR fragments (approx. 300-400bp) before proceeding to library preparation.

Library Preparation v1.0 - Setup

19 Thaw Pre-Combined UDI 96-well plate, purified PCR reaction, and KAPA Master Mix.

19.1 Generation of UDI plates.

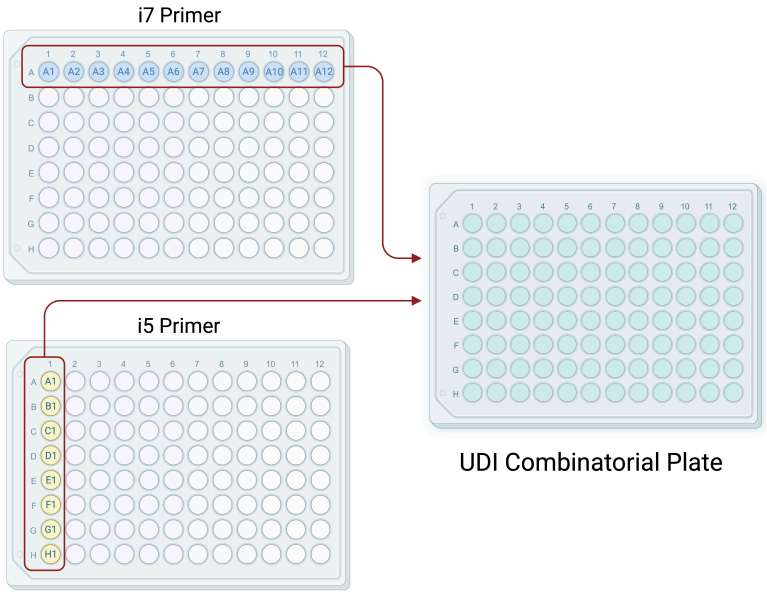


Figure. Generation of UDI (Unique Dual Index) plates. By ordering i5 (yellow) and i7 (blue) on plates in opposite orientation, UDI plates of combined i5 and i7 indexes (yellow + blue= green) can be made using a multichannel pipette to mix primers in a LoBind DNA plate. The column of i5 primers should be pipetted across all columns of the UDI plate and the i7 primer row pipetted across all rows of the plate.

The final concentration of the i5/i7 primer mix should be 10 micromolar. This can be done any number of ways, including diluting the 100 micromolar stock of each primer to 10 micromolar and then mixing.

- UDI Combinatorial Plates can me made well ahead of time and frozen for use.
- Typically we recommend making plates for a maximum of 10 indexing PCRs, thus if contamination should occur a new plate is accessed without wasting reagents.

Library Preparation v1.0 - Reaction Mixture

20 Calculate the amount of reagents needed with a 10% excess to account for pipette loss.

21 Combine KAPA HiFi HotStart ReadyMix 2X and water in a tube then aliquot equal volumes into 0.2mL microtube strips. Pipette 17.0 µL Master Mix into each well with a multichannel pipettor.

> Note
>
> You may use reverse pipetting technique to avoid bubbles and expedite this step.

	A	B
	Reagent	**Volume/Reaction**
	KAPA HiFi HotStart ReadyMix 2X	**12.5 µL**
	Molecular Grade Water	**4.5 µL**
	Total	**17.0 µL**

Library Preparation v1.0 - Addition of Unique Dual-Indexes and PfSMARRTer Amplicons

22 Spin UDI plate to ensure there is no liquid on the plate sealing foil.

23 Remove the foil seal carefully to avoid contamination.

24 Add 2.5 µL of the appropriate UDI to each well. **No need to mix yet.**

25 Seal stock UDI plate before opening and adding samples to prevent contamination.

26 Add 5.0 µL Combined and Purified PfSMARRTer amplicons. **Gently** mix by pipetting.

	A	B
	Reagent	**Volume/Reaction**
	Master Mix (described above)	**17.0 µL**
	UDI Primer	**2.5 µL**
	Combined & Purified PfSMARRT amplicons	**5.0 µL**
	Total	**24.5 µL**

Library Preparation v1.0 - Cycling Conditions

27 Program thermocycler with the following conditions for **8-12 cycles.**

	A	B	C	D
	Step	***Duration***	***Cycles***	***Temperature***
	Initial Activation Step	3 min	1	95° C
	Denaturation	***30 sec***	***8 - 12***	***95° C***
	Annealing	***30 sec***		***55° C***
	Extension	***30 sec***		***72° C***
	Final Extension	5 min	1	72° C
	Hold	Indefinite	1	4° C

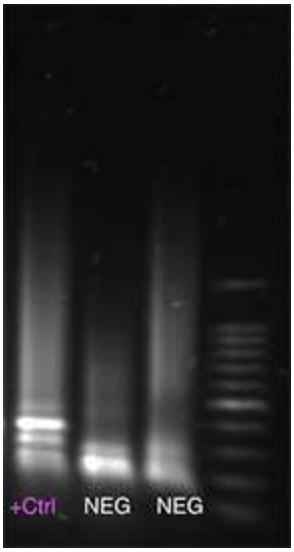
Check for contamination

28 Prior to proceeding to sample pooling into a final library, the libraries generated from positive and non-template controls should be checked for contamination.

28.1 It is expected that negative controls will have a detectable band on gel below the bands consistent with amplification products. Non-template control bands representing primer

dimer that should yield data when processed through bioinformatic analysis (See 30.2).

28.2

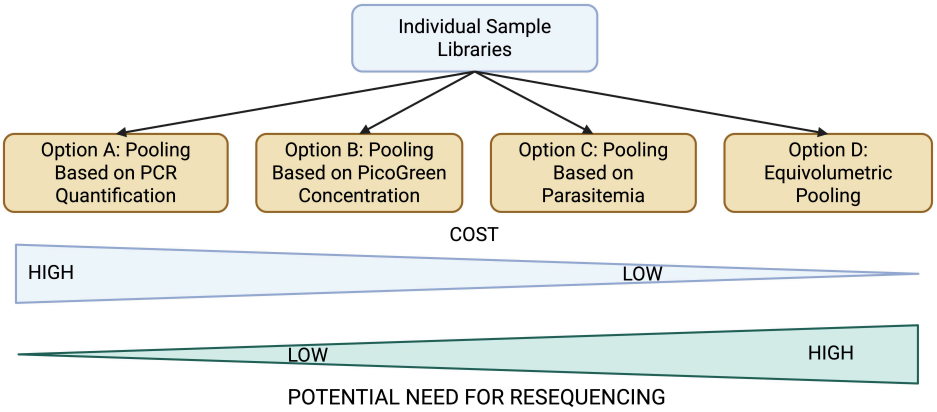


Gel electrophoresis showing positive control [DNA extracted from 1,000 Pf/uL dried blood spot using Chelex-Tween (first lane)] and negative controls (second and third lane). The gel was made with 1% agarose and 1X TBE buffer then run at 100 volts for 45 minutes. The 100bp ladder's (lane 4) smallest fragment is 100bp, and the bright band being 500 bp. (Promega 100bp Ladder)

28.3 If the negative controls have a bands consistent with target amplification bands, the plate/samples should be repeated and the source of contamination investigated.

Options for final pooling

29



Option A: Pooling Based on qPCR Quantification

30 Bring Speed-Beads to Room temperature (00:30:00).

- Mix fresh 80% Ethanol using 200 proof ethanol (100%, anhydrous) and molecular grade water.

31 Vortex Speed-Beads thoroughly before use.

32 Combine libraries with Speed-Beads at a 1:1 ratio (24.5 µL).

33 Allow the mixture to incubate at Room temperature for 00:10:00 .

34 Gently spin plate to collect contents at the bottom of wells.

35 Place the plate on a plate magnet and wait 00:02:00 - 00:03:00 for the solution to clear.

36 Aspirate the clear supernatant and discard.

37 Keeping the plate on the magnet, dispense 80% Ethanol into the wells to wash. Ethanol wash volume should be higher than the combined library and Speed-Bead mixture (e.g. 80 µL).

38 Incubate 00:00:30 then aspirate to wash.

39 Repeat for total of 2 washes.

40 Aspirate final drops of Ethanol with P20 pipette tips. Allow bead pellets to dry on the magnet for 00:01:00 - 00:03:00 .

- Dry beads to satin-matte finish, but not to the point of cracking. This happens quickly if ethanol has been aspirated completely.

41 Remove plate from magnet and dispense Elution Buffer onto each bead pellet. Mix by pipetting to resuspend DNA.

42 Allow the mixture to incubate at Room temperature for 00:05:00 .

43 Gently spin the plate to collect product in the bottom, then place on a plate magnet and wait 00:02:00 - 00:03:00 for the eluant to clear.

44 Remove eluant to a new LoBind plate.

45 Use a commercial Illumina Library Quantification Kit, following manufacturer's instructions, then pool samples based on calculated molarity.

46 The library pool is ready to prepare for sequencing.

Option B: Pooling Based on PicoGreen Concentration

1m 30s

47 Bring Speed-Beads to Room temperature (00:30:00).

- Mix fresh 80% Ethanol using 200 proof ethanol (100%, anhydrous) and molecular grade water.

48 Vortex Speed-Beads thoroughly before use.

49 Combine libraries with Speed-Beads at a 1:1 ratio (24.5 µL).

50 Allow the mixture to incubate at Room temperature for 00:10:00 .

51 Gently spin the plate to collect contents at the bottom of wells.

52 Place the plate on a plate magnet and wait 00:02:00 - 00:03:00 for the solution to clear.

53 Aspirate the clear supernatant and discard.

54 Keeping the plate on the magnet, dispense 80% Ethanol into the wells to wash. Ethanol wash volume should be higher than the combined library and Speed-Bead mixture (e.g. 80 µL).

55 Incubate 00:00:30 then aspirate to wash.

56 Repeat for total of 2 washes.

57 Aspirate final drops of Ethanol with P20 pipette tips. Allow bead pellets to dry on the magnet for 00:00:30 - 00:01:00 . 1m 30s

- Dry beads to satin-matte finish, but not to the point of cracking. This happens quickly if ethanol has been aspirated completely.

58 Remove plate from magnet and dispense 25 µL Elution Buffer onto each bead pellet. Mix by pipetting to resuspend DNA.

59 Allow the mixture to incubate at Room temperature for 00:05:00 .

60 Gently spin the plate to collect product in the bottom of wells, then place on a plate magnet and wait 00:02:00 - 00:03:00 for the eluant to clear.

61 Remove eluant to a new LoBind plate.

62 Quantify 2 µL of amplified libraries in 48 µL of 1X PicoGreen reagent.

- If reagent is not already provided at 1X, mix 1:200 dye to buffer to create a working solution.
- If you are quantifying an entire 96-well plate of samples, a second plate will be necessary for assay standards.

63 Add 2 µL of each sample to a black, flat bottomed polystyrene assay plate.

- The 90-degree angle where the well wall meets the flat bottom holds this droplet nicely.

64
- Add 2 µL of each standard to the appropriate wells.
- Standards 1-8 in duplicate = 16 wells required.

65 Fill each sample and standard wells with 48 µL 1X PicoGreen Reagent.

- Pipette to mix thoroughly, careful not to introduce bubbles.

66 Cover plate with optical film and incubate for 00:02:00 at Room temperature before assaying. Protect from light during incubation.

67 Place the prepared quantitation plate into the multimode plate reader.

- Plate reader should be set up to analyze Fluorescence Intensity (FI) with an excitation wavelength around 480nm and an emission wavelength around 580nm.

68 Calculate each sample's concentration by creating a standard curve and plotting unknown fluorescence values. Divide resulting value by two to report nanograms per microliter.

68.1 Negative controls can have detectable DNA by PicoGreen in the form of dimers (Step 30.2). To determine the concentration of library in order to pool equimolar volume of

each sample, one should use a baseline (averaged NTC concentration) subtracted from the concentration for each sample to determine pooling volumes.

69 The library pool is ready to prepare for sequencing.

Option C: Pooling Based on Parasitemia

70 Determine estimated parasitemia of samples by microscopy or real-time PCR.

71 Bin samples by parasitemia quartile (or other division that makes sense based on parasitemia distribution), recording the number of samples per subpool.

72 Mix 2uL of each sample for each quartile or subpool into a separate LoBind 1.5mL tube.

72.1 You will end up with 4 or more subpools depending on how you group your samples.

73 Bring Speed-Beads to Room temperature (00:30:00).

- Mix fresh 80% Ethanol using 200 proof ethanol (100%, anhydrous) and molecular grade water.

74 Vortex Speed-Beads thoroughly before use.

75 Combine final pooled libraries with Speed-Beads at a 1:1 ratio in the LoBind 1.5mL tube.

76 Allow the mixture to incubate at Room temperature for 00:10:00 .

77 Gently spin tube to collect contents at the bottom.

78 Place the tube on a tube magnet and wait 00:02:00 - 00:03:00 for the solution to clear.

79 Aspirate clear supernatant and discard.

80 Keeping the tube on the magnet, dispense 80% Ethanol into the tube to wash. Ethanol wash volume should be higher than library and Speed-Bead mixture.

81 Incubate 00:00:30 then aspirate to wash.

82 Repeat for total of 2 washes.

83 Aspirate final drops of Ethanol with P20 pipette tips. Allow bead pellets to dry on the magnet for 00:01:00 - 00:03:00 .

- Dry beads to satin-matte finish, but not to the point of cracking.

84 Remove tube from magnet and dispense Elution Buffer onto each bead pellet. Mix by pipetting to resuspend DNA.

85 Allow the mixture to incubate for 00:05:00 .

86 Gently spin the tube to collect product in the bottom, then place on a tube magnet and wait 00:02:00 - 00:03:00 for the solution to clear.

87 Remove fluid to a new LoBind tube.

88 Quantify each subpool using a commercial Illumina Library Quantification Kit.

89 Combine the subpools using the library concentration weighted by the number of samples within each.

90 Quantify library pool using Qubit dsDNA HS reagent according to the manufacturers protocol.

91 The library pool is ready to prepare for sequencing.

Option D: Equivolumentric Pooling

92 Pool 2ul of each library into a LoBind 1.5mL tube. *This option does not require SPRI purification prior to pooling libraries.*

93 Bring Speed-Beads to Room temperature (00:30:00).

- Mix fresh 80% Ethanol using 200 proof ethanol (100%, anhydrous) and molecular grade water.

94 Vortex Speed-Beads thoroughly before use.

95 In a LoBind 1.5mL tube, combine final pooled libraries with Speed-Beads at a 1:1 ratio.

96 Allow the mixture to incubate at Room temperature for 00:10:00 .

97 Gently spin tube to collect contents at the bottom.

98 Place the tube on a tube magnet and wait 00:02:00 - 00:03:00 for the solution to clear.

99 Aspirate clear supernatant and discard.

100 Keeping the tube on the magnet, dispense 80% Ethanol into the tube to wash. Ethanol wash volume should be higher than library and Speed-Bead mixture.

101 Incubate 00:00:30 then aspirate to wash.

102 Repeat for total of 2 washes.

103 Aspirate final drops of Ethanol with P20 pipette tips. Allow bead pellets to dry on the magnet for 00:01:00 - 00:03:00 .

- Dry beads to satin-matte finish, but not to the point of cracking.

104 Remove tube from magnet and dispense Elution Buffer onto each bead pellet. Mix by pipetting to resuspend DNA.

105 Allow the mixture to incubate for 00:05:00 .

106 Gently spin the tube to collect product in the bottom, then place on a tube magnet and wait 00:02:00 - 00:03:00 for the solution to clear.

107 Remove fluid to a new LoBind tube.

108 Quantify library pool using Qubit dsDNA HS reagent according to the manufacturers protocol.

109 The library pool is ready to prepare for sequencing.