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Version 1

Pf-SMARRTer- *Plasmodium falciparum* Streamlined Multiplex Antimalarial Resistance and Relatedness Testing v1.13 V.1

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Protocol status: Working

We use this protocol and it's working

Created: February 14, 2025

Last Modified: March 04, 2025

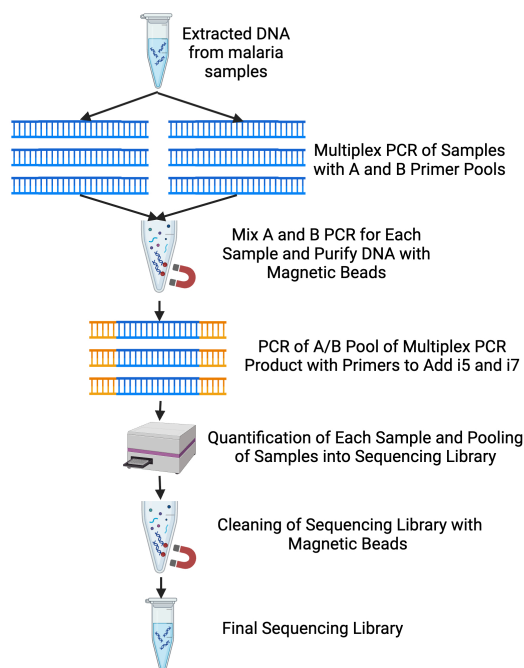
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Abstract

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

Guidelines

Infectious Disease Epidemiology and Ecology Lab University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

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 <i>k13</i> 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 <i>mdr184</i> , <i>crt</i> , <i>k13 622l</i> and <i>HeOME</i> - 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 <i>dhfr 164</i> and <i>pfmdr1 1246</i> - Removal of <i>pfmdr1 1034</i>



	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	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAATTGTACTAAACCTATAGATACTAATGATAATATTATAGG
		MDR1_184_F_v2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGAGTTCAGGAATTGGTACGA
		MDR1_184_R_v2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCCTCTTCTATAATGGACATGGT
		MDR1_1246_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAAAATGTAAATGAATTTTCAAACCAATCTGGA
		MDR1_1246_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATAGCAGCAAACCTACTAACA CGTTTAA
	dhfr	DHFR_51_59_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGAGGTTTTTAATAACTACACATTTAGAGGTCT
		DHFR_51_59_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTATCATTTACATTATCCACAGTTCTTTGTT
		DHFR_108/164_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAAAATTACAAAATGTTGTAGTTATGGGAAGAA
		DHFR_108/164_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCTAAAAATTCTTGATAAACAACGGAACCT
	dhps	DHPS_437_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGAAATGATAAATGAAGGTGCTAGTGT
		DHPS_437_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAATACAGGTACTACTAAATCTCTTCACTAATTTTT
		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	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTTGTGTATTTATTACAACATTTGATCATTC
	k13	K13-A-432-466_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAAAGTGAAGCCTTGTTGAAAGAAG
		K13-A-432-466_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTACACATACGCCAGCATTGTG
		K13-B-461-531_F2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGATGGTGTAGAATATTTAAATTCGATG
		K13-B-461-531_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTACCATTTGACGTAACACCA
		K13-C-513-570_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTTGAAACTGAGGTGTATGATCG
		K13-C-513-570_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTGATGATCTAGGGGTATTC
		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	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAGAAATTTATCAGAGTTACA
		HeOME-B F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTTATCCTTATCATTATTTCCATCATTCTGG
		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	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATCTTTTTCTGTTGTATGTGCATAATCA
		HeOME-F R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAACATAATTCTAATGATATTGACCTTGTGCA
		HeOME-G F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACATTCACACAAATAGAAAAATCTTCATTTTTTC
		HeOME-G R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATCAATAATCAAAATCATGATAACAACCAATTT
		HeOME-H F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAATAACTTAAATAAAAAATATGGACGGCTCC
		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
	CAAGCAGAAGACGGCAT ACGAGAT	CGCTCAG TTC	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATACGAGATCGCTCAGTTC GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TATCTGAC CT	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATACGAGATTATCTGACCT GTCTCGTGGGCTCGG



	A	B	C	D
	CAAGCAGAAGACGGCAT ACGAGAT	TCGGATG TCG	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTCGGATGTCTG GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	CTTATGG AAT	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATCTTATGGAATG TCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TCCTATTG TG	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTCCTATTGTG GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	GCGCGAT GTT	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATGCGCGATGTT GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	AGAGCAC TAG	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATAGAGCACTAG GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TGCCTTG ATC	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTGCCTTGATC GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	CTACTCA GTC	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATCTACTCAGTC GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TCGTCTG ACT	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTCGTCTGACT GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	GAACATA CGG	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATGAACATACGG GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	CCTATGA CTC	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATCCTATGACTC GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TAATGGC AAG	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTAATGGCAAG GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	GTGCCGC TTC	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATGTGCCGCTTC GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	CGGCAAT GGA	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATCGGCAATGGA GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	GCCGTAA CCG	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATGCCGTAACCG GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	AACCATT CTC	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATAACCATTCTC GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	GGTTGCC TCT	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATGGTTGCCTCT GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	CTAATGAT GG	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATCTAATGATGG GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TCGGCCT ATC	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTCGGCCTATC GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	AGTCAAC CAT	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATAGTCAACCAT GTCTCGTGGGCTCGG



	A	B	C	D
	CAAGCAGAAGACGGCAT ACGAGAT	GAGCGCA ATA	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATGAGCGCAATA GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	AACAAGG CGT	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATAACAAGGCGT GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	GTATGTAG AA	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATGTATGTAGAAG TCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TTCTATG GTT	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTTCTATGGTTG TCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	CCTCGCA ACC	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATCCTCGCAACC GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TGGATGC TTA	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTGGATGCTTA GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	ATGTCGT GGT	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATATGTCGTGGT GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	AGAGTGC GGC	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATAGAGTGCGGC GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TGCCTGG TGG	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTGCCTGGTGG GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TGCGTGT CAC	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTGCGTGTAC GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	CATACAC TGT	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATCATACACTGT GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	CGTATAAT CA	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATCGTATAATCAG TCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TACGCGG CTG	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTACGCGGCTG GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	GCGAGTT ACC	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATGCGAGTTACC GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TACGGCC GGT	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTACGGCCGGT GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	GTCGATT ACA	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATGTCGATTACA GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	CTGTCTG CAC	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATCTGTCTGCAC GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	CAGCCGA TTG	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATCAGCCGATTG GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TGACTAC ATA	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTGACTACATAG TCTCGTGGGCTCGG

	A	B	C	D
	CAAGCAGAAGACGGCAT ACGAGAT	ATTGCCG AGT	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATATTGCCGAGT GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	GCCATTA GAC	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATGCCATTAGAC GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	GGCGAGA TGG	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATGGCGAGATGG GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TGGCTCG CAG	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTGGCTCGCAG GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TAGAATAA CG	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTAGAATAACG GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TCCATGT TGC	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTCCATGTTGC GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	TATCCAG GAC	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATTATCCAGGAC GTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCAT ACGAGAT	AGTGCCA CTG	GTCTCGTGG GCTCGG	CAAGCAGAAGACGGCATAACGAGATAGTGCCACTG GTCTCGTGGGCTCGG

Table 1.4 - I5 index

	A	B	C	D
	Beginning	Index	End	To order
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	AATGATACGGCGACCACCG AGATCTACAC	CTACAA GATA	TCGTGCGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACCTACA AGATATCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	TACGTTT ATT	TCGTGCGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACTACGT TCATTTTCGTGCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	TGCCTG GTGG	TCGTGCGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACTGCC TGGTGGTCGTGCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	TCCATC CGAG	TCGTGCGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACTCCAT CCGAGTCGTGCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	GTCCAC TTGT	TCGTGCGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACGTCC ACTTGTTCGTGCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	TGGAAC AGTA	TCGTGCGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACTGGA ACAGTATCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	CCTTGT AAT	TCGTGCGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACCCTT GTTAATTCGTGCGGCAGCGTC



	A	B	C	D
	AATGATACGGCGACCACCG AGATCTACAC	GTTGATA GTG	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACGTTGA TAGTGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	ACCAGC GACA	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACACCA GCGACATCGTCGGCAGCGTC
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	AATGATACGGCGACCACCG AGATCTACAC	GTGTGG CGCT	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACGTGTG GCGCTTCGTCGGCAGCGTC
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	AATGATACGGCGACCACCG AGATCTACAC	TCGGCA GCAA	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACTCGG CAGCAATCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	CTAATGA TGG	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACCTAAT GATGGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	GGTTGC CTCT	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACGGTT GCCTCTTCGTCGGCAGCGTC
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	AATGATACGGCGACCACCG AGATCTACAC	CTGTGTT AGG	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACCTGTG TTAGGTCGTCGGCAGCGTC
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	AATGATACGGCGACCACCG AGATCTACAC	CTAACT GTAA	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACCTAAC TGTAATCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	GGCGAG ATGG	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACGGCG AGATGGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	AATAGAG CAA	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACAATAG AGCAATCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	TCAATCC ATT	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACTCAAT CCATTTTCGTCGGCAGCGTC



	A	B	C	D
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	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 AATATTTTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	GATCAC CGCG	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACGATCA CCGCGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	TACCATC CGT	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACTACCA TCCGTTTCGTCGGCAGCGTC
	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
	AATGATACGGCGACCACCG AGATCTACAC	GTCCTG GATA	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACGTCC TGGATATCGTCGGCAGCGTC
	AATGATACGGCGACCACCG AGATCTACAC	CAGTGG CACT	TCGTCGGCA GCGTC	AATGATACGGCGACCACCGAGATCTACACCAGT GGCACTTCGTCGGCAGCGTC

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**
- 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>)

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	12.5 μl
Primer Pool+	2.5 μl
Template DNA*	2.0 μl
PCR grade water	8.0 μl

*The "primer pool mix" represents one for pool 1 that is separate from pool 2.

*Template DNA volume can be adjusted depending on the concentration of DNA by changing the amount of PCR grade water in the reaction.

- 3.1 All reagents, PCR master mix, PCR reagents and PCR mixes 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 name and which primer pool (A or B) is being used in the PCR.

- 5 Combine the master mix and sample DNA, seal caps/strips/plates, and place in a thermocycler 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

50m 30s

- 6 Bring beads to  Room temperature ( 00:30:00).
- 7 In a 96 well LoBind PCR plate, combine the entire P1 and P2 PCR reaction 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 .
- 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.

30m



10m





- 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 clear supernatant and discard.
- 12 Dispense 120 μ L 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 EtOH with P20 pipette tips. Allow bead pellets to dry on the magnet for 00:01:00 - 00:03:00 . 4m
- 14 Remove Plate from magnet.
- 15 Dispense 25 μ L 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.
- 17 Place the plate on a 96 well magnetic block and wait 00:02:00 - 00:03:00 for the solution to clear. 3m
- 18 Transfer fluid to a new LoBind PCR plate for further processing.

Spot-quantitation

- 19 Using Qubit dsDNA HS reagent, spot-quantify three samples to ensure amplicon DNA was not lost during purification following the manufacturer's protocol.
- 20 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

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

21.1 Generation of UDI index plates.

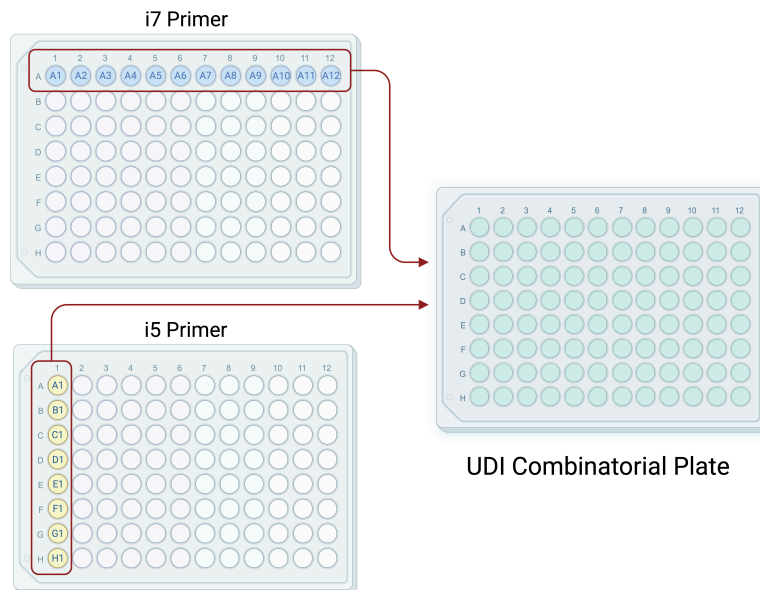


Figure. Generation of UDI Combinatorial Index Plates. By ordering i5 (yellow) and i7 (blue) on plates in opposite orientation, UDI combinatorial plates of unique combinations of i5 and i7 indexes (yellow + blue = green) can be generated using a multichannel pipette to mix primers in a LoBind DNA plate. The column of i5 primers should be pipetted across all columns of the UDI Combinatorial 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 be 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

- 22 Calculate the amount of reagents needed with a 10% excess to account for pipette loss.
- 23 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 PfSMARRT Amplicons

- 24 Spin UDI plate to ensure there is no liquid on the plate sealing foil.
- 25 Remove the foil seal carefully to avoid contamination.
- 26 Add  2.5 µL of the appropriate UDI to each well. **No need to mix yet.**
- 27 Seal stock UDI plate before opening and adding samples to prevent contamination.
- 28 Add  5.0 µL Combined and Purified PfSMARRT 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 DNA	5.0 µL
Total	24.5 µL

Library Preparation v1.0 - Cycling Conditions

29 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 cycles - 12 cycles	95° C
Annealing	30 sec		55° C
Extension	30 sec		72° C
Final Extension	5 min	1	72° C
Hold	Indefinite	1	4° C

Note

There is no need to perform a PCR-clean up step after Library Preparation PCR. Proceed directly to quantification and pooling.

Library Preparation v1.0 - PicoGreen Quantification on Multimode Plate Reader

2m

30 Quantify  2 µL of amplified libraries in 48µL 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.

31 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.

32 Add  2 μL of each standard to the appropriate wells.



- Standards 1-8 in duplicate=16 wells required.

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



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

34 Cover plate with optical film and incubate for  00:02:00 at  Room temperature before assaying.

2m



35 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.

36 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.

37 **To pool equimolar volumes, divide the total nanograms of DNA desired in the pool by the calculated DNA concentration for each sample,**

For example:

- A pool is needed containing 300 nanograms of each library.
- The library DNA concentration is 64.25 ng/ μL .
- Volume of library to add to pool (x)=300/64.25 $\rightarrow$ x = 4.67 μL

Nanograms per library can be adjusted based on sample concentration.
Lower sample concentrations will require lower total nanograms pooled.

Cleanup of final pooled libraries

59m 30s



- 38 Bring beads to Room temperature (00:30:00). 30m

▪ Mix fresh 80% Ethanol using 200 proof ethanol (100%, anhydrous) and molecular grade water.
- 39 Vortex Speed-Beads thoroughly before use.
- 40 In a LoBind 1.5mL tube, combine final pooled libraries with Speed-Beads at a 1:1 ratio.
- 41 Allow the mixture to incubate at Room temperature for 00:10:00 . 10m
- 42 Gently spin tube to collect contents at the bottom.
- 43 Place the tube on a tube magnet and wait 00:02:00 - 00:03:00 for the solution to clear. 5m
- 44 Aspirate clear supernatant and discard.
- 45 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.
- 46 Incubate 00:00:30 then aspirate to wash. 30s
- 47 Repeat for total of 2 washes.
- 48 Aspirate final drops of EtOH with P20 pipette tips. Allow bead pellets to dry on the magnet for 00:01:00 - 00:03:00 . 4m
- Dry beads to satin-matte finish, but not to the point of cracking.
- 49 Remove tube from magnet and dispense Elution Buffer onto each bead pellet. Mix by pipetting to resuspend DNA.



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

5m



51 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.

5m



52 Remove fluid to a new LoBind tube.

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

54 The library pool is ready to prepare for sequencing.