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IDEEL- UNC Implemented 4CAST Modification (Nextera Library Preparation Protocol) V.1

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We use this protocol and it's working

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Abstract

This protocol details the 4CAST modification of Nextera Library Preparation.

Guidelines

Overview:

This protocol is designed for use with the current 4CAST amplicon panel. The original publication of this protocol is: LaVerriere, E., Schwabl, P., Carrasquilla, M., Taylor, A. R., Johnson, Z. M., Shieh, M., et al. (2022). Design and implementation of multiplexed amplicon sequencing panels to serve genomic epidemiology of infectious disease: A malaria case study. Mol Ecol Resour 22, 2285–2303.

Portions of this protocol are taken verbatim from Supplementary Protocol S1 from this manuscript.

PCR assays are “Nextera-Adapted” by adding standardized, identical oligo segments onto existing primer sequences.

For example:

Non-Nextera-Adapted Primer→
Forward primer: CCATCAGGGAAATGTCCAGT
Reverse primer : TTTCCTGCATGTCTTGAACA
Forward primer addition: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
Reverse primer addition: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

Nextera-Adapted Primer:
Forward: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCATCAGGGAAATGTCCAGT
Reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTTCCTGCATGTCTTGAACA

PCR-based library preparation allows for a significant reduction in time to complete libraries, reduces the consumables required, and lessens the impact of library preparation on technicians. Pre-combined 96-well Unique Dual Index plates are utilized in combination with standardized barcoding worksheets to simplify the generation of final library indexing manifests.

4CAST Primers

Primer	To Order
4-CSP-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTAAGGAACAAGAAGGATAATACCA
4-CSP-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAATGACCCAAACCGAAATG
4-TRAP-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCCAGCACATGCGAGTAAAG
4-TRAP-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAACCCGAAAATAAGCACGA
4-SERA-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTACTTTCCCTTGCCCTTGTG
4-SERA-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCACTACAGATGAATCTGCTACAGGA
4-AMA-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCATCAGGGAAATGTCCAGT
4-AMA-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTTCCTGCATGTCTTGAACA

I7 Index primers

	Beginning	Index	End	To order
	CAAGCAGAAGACGGCATACGAGAT	CGCTCAGTTC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATCGCTCAGTTCGTCTCGTGGGCTCGG
	CAAGCAGAAGACGGCATACGAGAT	TATCTGACCT	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTATCTGACCTGTCTCGTGGGCTCGG
	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
	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
	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

I5 Index Primers

	A	B	C	D
	Beginning	Index	End	To order
	AATGATACGGCGACCACCGAGATCTACAC	TCGTGGAGCG	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACTCGTGGAGCGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	CTACAAGATA	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACCTACAAGATATC

	A	B	C	D
				GTCGGCAG CGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	TACGTTCATT	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACTAC GTTCATTT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	TGCCTGGTGG	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACTGC CTGGTGGT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	TCCATCCGAG	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACTCC ATCCGAGT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	GTCCACTTGT	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACGTC CACTTGTT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	TGGAACAGTA	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACTGG AACAGTAT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	CCTTGTTAAT	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACCCT TGTTAATTC GTCGGCAG CGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	GTTGATAGTG	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACGTT GATAGTGT CGTCGGCA GCGTC

	A	B	C	D
	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
	AATGATACGGCGACCA	TCGGCAGCAA	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATC

	A	B	C	D
	CCGAGATC TACAC			TACACTCG GCAGCAAT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	CTAATGATGG	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACCTA ATGATGGT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	GGTTGCCTCT	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACGGT TGCCTCTT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	CGCACATGGC	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACCGC ACATGGCT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	GGCCTGTCCT	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACGGC CTGTCCTT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	CTGTGTTAGG	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACCTG TGTTAGGT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	TAAGGAACGT	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACTAA GGAACGTT CGTCGGCA GCGTC

	A	B	C	D
	AATGATACGGCGACCACCGAGATCTACAC	CTAACTGTAA	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACCTAACTGTAATCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	GGCGAGATGG	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACGGCGAGATGGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	AATAGAGCAA	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACAATAGAGCAATCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	TCAATCCATT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACTCAATCCATTTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	TCGTATGCGG	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACTCGTATGCGGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	TCCGACCTCG	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACTCCGACCTCGTCGTCGGCAGCGTC
	AATGATACGGCGACCACCGAGATCTACAC	CTTATGGAAT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACCTTATGGAATTCGTCGGCAGCGTC
	AATGATACGGCGACCA	GCTTACGGAC	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATC

	A	B	C	D
	CCGAGATC TACAC			TACACGCT TACGGACT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	GAACATACGG	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACGAA CATACGGT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	GTCGATTACA	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACGTC GATTACATC GTCGGCAG CGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	ACTAGCCGTG	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACACT AGCCGTGT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	AAGTTGGTGA	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACAAG TTGGTGAT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	TGGCAATATT	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACTGG CAATATTTC GTCGGCAG CGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	GATCACCGCG	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACGAT CACCGCGT CGTCGGCA GCGTC

	A	B	C	D
	AATGATAC GGCGACCA CCGAGATC TACAC	TACCATCCGT	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACTAC CATCCGTT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	GCTGTAGGAA	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACGCT GTAGGAAT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	CGCACTAATG	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACCGC ACTAATGT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	GACAACTGAA	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACGAC AACTGAAT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	AGTGGTCAGG	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACAGT GGTCAGGT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	CCATCTCGCC	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACCCA TCTCGCCT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	CTGCGAGCCA	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACCTG CGAGCCAT CGTCGGCA GCGTC
	AATGATAC GGCGACCA	CGTTATTCTA	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC

	A	B	C	D
	CCGAGATC TACAC			TACACCGT TATTCTATC GTCGGCAG CGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	GCAACATGGA	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACGCA ACATGGAT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	GTCCTGGATA	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACGTC CTGGATAT CGTCGGCA GCGTC
	AATGATAC GGCGACCA CCGAGATC TACAC	CAGTGGCACT	TCGTCGGC AGCGTC	AATGATAC GGCGACCA CCGAGATC TACACCAG TGGCACTT CGTCGGCA GCGTC

Materials

Consumables:

1. P200 pipettor and tips
2. P20 pipettor and tips
3. 25µL reagent reservoir (3)
4. 96-well PCR plates

Reagents:

1. 80% Ethanol
2. AmpureXP beads
3. KAPA HiFi HotStart ReadyMix 2X
4. Molecular Grade Water (MGW)
5. Pre-Combined UDI 96-well plate @ 10µM each index (i7 and i5 Index primers)
6. 4CAST primer

4CAST Primer Mix Preparation

1 Combine 10 µL of each primer (forward and reverse for CSP, AMA1, SERA2, and TRAP) at [M] 100 micromolar (µM) into 1.5mL Eppendorf tube.

1.1 Store primer cocktail at -20 °C.

1.2 Volume will need to be modified if more than 96 samples are being prepared at once.

2 Perform a 1:1 dilution with MGW to achieve a working concentration of [M] 6.250 micromolar (µM) of each primer.

2.1 For primer mix with 10 µL of each primer, use 80 µL of primer mix and 80 µL dH_2O.

PCR1

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

4 Prepare the PCR1 cocktail using the following quantities for each sample being prepared and pipet 6.5 µL of mix into each well of a 96-well PCR plate.

	A	B
	REAGENT	**VOLUME/REACTION**
	KAPA HiFi HotStart ReadyMix 2X	5.0 µL
	Primer Cocktail	1.5 µL
	Total MM Volume	6.5 µL

5 Add 4.0 µL sample genomic DNA (or negative/positive templates) to each well.

5.1 Recommended to place controls randomly throughout the plate.

6 Place the plate on a thermocycler and program with the following conditions:

Step	# cycles	Temp C	Time
Activation	1	95	3 min.
-Denaturation	30	98	20 sec.
-Annealing		57	15 sec.
-Extension		62	30 sec.
Final Extension	1	72	1 min.
hold	1	4	forever

Setup for Library Preparation PCR: Preparation of Materials

7 Generation of UDI plates is described at Step 19 at dx.doi.org/10.17504/protocols.io.e6nvwbwy7vmk/v2.

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

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

10 **Record the combinatorial index plate used (e.g. UDI-A1, UDI-C3, UDI-E5).**

Setup for Library Preparation PCR: Preparing UDI Plate

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

12 Remove foil from desired UDI wells row-by-row or column-by-column, using a razor to carefully score between wells.

12.1 Use the edge of the razor to lift the foil before gently peeling the foil off.

12.2 This method allows for easy tracking of what indexes have been added and helps limit contamination of index plates.

13 Dilute UDI in plate as necessary to reach a concentration of [M] 10 micromolar (µM) .

13.1 Add 40 µL Tris with no EDTA (if possible) for dilution from UDI plates.

13.2 Perform dilution in the UDI plate.

14 Add 2.2 µL of the appropriate UDI to each well. **No need to mix yet**.

14.1 Record which well of the UDI plate was used for each sample.

15 Seal UDI plate with a fresh piece of foil immediately after use.

Library Preparation PCR

16 Calculate the amount of master mix reagents needed with a 10% excess to account for pipette loss. (Use tool **here** for calculations)

17 Prepare LP-PCR plate for indexing samples with the following reaction volumes.

	A	B
	REAGENT	**VOLUME/REACTION**
	KAPA HiFi HotStart ReadyMix 2X	5.0 µL
	Molecular H2O	2.0 µL
	Unique Dual Index	2.2 µL

	A	B
	Total MM Volume	9.2 µL

18 Add 3.0 µL of PCR 1 product to each well.

	A	B	C	D
	STEP	**# CYCLES**	**TEMP C**	**TIME**
	Activation	1	95	1 min.
	-Denaturation	15	95	15 sec.
	-Annealing		55	15 sec
	-Extension		72	30 sec
	Final Extension	1	72	1 min.
	hold	1	4	forever

19 Place the plate on a thermocycler and program with the following conditions:

Note

Sample can be frozen at -20 °C at this point.

Sample pooling and double-sided clean-up/size selection 20m

20

Note

Equilibrate beads to Room temperature for 00:30:00 prior to use; take Tapestation reagents out of 4°C to come to room temperature prior to use (if using).

Combine approximately 8 µL of each sample into a 2.0mL Eppendorf tube to create the sample pool.

20.1 If completing with less than 13 samples, can adjust volume of each sample accordingly (ex: use 10 µL of each sample) to achieve an adequate sample pool volume.

20.2 Mix gently and spin down.

21 Perform bead clean-up using AmpureXP beads for size selection.

21.1 Transfer 100 µL combined sample into a PCR tube and add 55 µL beads, mixing well.

21.2 Incubate at Room temperature for 00:05:00 . 5m

21.3 Place on a magnetic rack for 00:05:00 . 5m

21.4 **KEEP** and remove supernatant from beads and transfer into a new PCR tube.

21.5 Add 20 µL of beads, mixing well and incubating at Room temperature for 00:05:00 . 5m

21.6 Place on the magnetic rack for 00:03:00 . 3m

21.7 Discard supernatant and wash twice with 80% ethanol while still on rack.

- Use approximately 150 µL for each wash.

21.8 Elute in 30 µL [M] 10 millimolar (mM) Tris-HCl pH 8.0, incubate for 00:02:00 , place on the magnetic rack, and allow the pellet to form (about 3 minutes). 2m

- If using stock Tris-HCl, concentration is typically 1M; therefore, dilute down to [M] 10 millimolar (mM) with 1 µL of Tris-HCl into 99 µL molecular grade water.

21.9 **Keep** and remove supernatant and transfer to a 1.5mL Eppendorf tube.

21.10 Add 2.5 µL [M] 10.0 millimolar (mM) Tris-HCl containing 0.05% Tween-20 for final suspension.

QC/library selection for sequencing

22 A full qPCR quantification is not necessary for 4CAST.

23 We recommend:

23.1 Qubit

23.2 Tapestation

24 QC can be done by the core or sequencing facility.