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IDEEL- UNC Implementation of Targeted csp Sequencing- MIP to Nanopore V.1

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IDEEL

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

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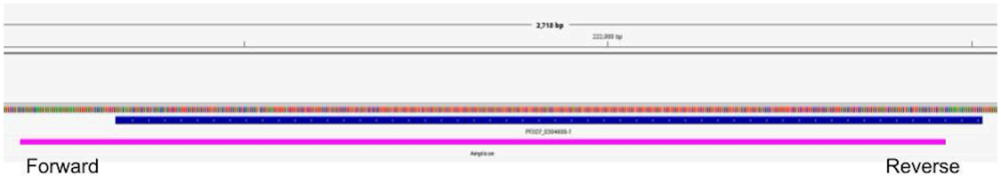
Keywords: PCR2 primer preparation, Pooling and Cleanup, Nanopore library preparation, Adaptor ligation, Nanopore sequencing, nanopore this protocol detail, amplicon generation through pcr2 primer preparation, nanopore library preparation, pcr2 primer preparation, nanopore, sequencing, amplicon generation, derived amplicon generation, unc implementation, mip, csp

Abstract

This protocol details the IDEEL-UNC targeted csp sequencing workflow, from MIP-derived amplicon generation through PCR2 primer preparation, pooling, cleanup, Nanopore library preparation, adaptor ligation, and sequencing.

Guidelines

Location of amplicon relative to *csp*:



Alignment of reverse primer

```
XM_001351086.1      ATGATGAGAAAATTAGCTATTTTATCTGTTTCTTCCTTTTTATTTGTTGAGGCCTTATTC
reverse             ------------------------------------------------------CCTTATTC
                                                                         ********

XM_001351086.1      CAGGAATACCAGTGCTATGGAAGTTCGTCAAACACAAGGGTTCTAAATGAATTAAATTAT
reverse             CAGGAATACCAGTGC---------------------------------------------
                    ***************

XM_001351086.1      GATAATGCAGGCACTAATTTATATAATGAATTAGAAATGAATTATTATGGGAAACAGGAA
reverse             ------------------------------------------------------------

XM_001351086.1      AATTGGTATAGTCTTAAAAAAAAATAGTAGATCACTTGGAGAAAATGATGATGGAAATAAC
reverse             ------------------------------------------------------------

XM_001351086.1      GAAGACAACGAGAAATTAAGGAAACCAAAACATAAAAAATTAAAGCAACCAGCGGATGGT
reverse             ------------------------------------------------------------

XM_001351086.1      AATCCTGATCCAAATGCAAACCCAAATGTAGATCCCAATGCCAACCCAAATGTAGATCCA
reverse             ------------------------------------------------------------

XM_001351086.1      AATGCAAACCCAAATGTAGATCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCA
reverse             ------------------------------------------------------------

XM_001351086.1      AATGCAAACCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCA
reverse             ------------------------------------------------------------

XM_001351086.1      AATGCAAACCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCAAATGCAAACCCA
reverse             ------------------------------------------------------------

XM_001351086.1      AATGCAAACCCCAATGCAAATCCTAATGCAAACCCAAATGCAAACCCAAACGTAGATCCT
reverse             ------------------------------------------------------------

XM_001351086.1      AATGCAAATCCAAATGCAAACCCAAACGCAAACCCCAATGCAAATCCTAATGCAAACCCC
reverse             ------------------------------------------------------------

XM_001351086.1      AATGCAAATCCTAATGCAAATCCTAATGCCAATCCAAATGCAAATCCAAATGCAAACCCA
reverse             ------------------------------------------------------------

XM_001351086.1      AACGCAAACCCCAATGCAAATCCTAATGCCAATCCAAATGCAAATCCAAATGCAAACCCA
reverse             ------------------------------------------------------------

XM_001351086.1      AATGCAAACCCAAATGCAAACCCCAATGCAAATCCTAATAAAAACAATCAAGGTAATGGA
reverse             ------------------------------------------------------------

XM_001351086.1      CAAGGTCACAATATGCCAAATGACCCAAACCGAAATGTAGATGAAAATGCTAATGCCAAC
reverse             ------------------------------------------------------------

XM_001351086.1      AGTGCTGTAAAAAATAATAATAACGAAGAACCAAGTGATAAGCACATAAAAGAATATTTA
reverse             ------------------------------------------------------------

XM_001351086.1      AACAAAATACAAAATTCTCTTTCAACTGAATGGTCCCCATGTAGTGTAACTTGTGGAAAT
reverse             ------------------------------------------------------------

XM_001351086.1      GGTATTCAAGTTAGAATAAAGCCTGGCTCTGCTAATAAACCTAAAGACGAATTAGATTAT
reverse             ------------------------------------------------------------

XM_001351086.1      GCAAATGATATTGAAAAAAAAAATTTGTAAAATGGAAAAATGTTCCAGTGTGTTTAATGTC
reverse             ------------------------------------------------------------

XM_001351086.1      GTAAATAGTTCAATAGGATTAATAATGGTATTATCCTTCTTGTTCCTTAATTAG
reverse             ------------------------------------------------------
```

Materials

- Molecular Grade Water (MGW)
- Primers
  - sp-rtss-repeat_v4_F_MIP GACTCGCCAAGCTGAAGNNNNTCGCAAACGTAATTAAATATTCACAAA
  - csp-rtss-repeat_v4_R_MIP ACGTGTGCTCTTCCGATCTNNNNCCTTATTCCAGGAATACCAGTGC
- GoTaq® Long PCR Master Mix, 2X **Promega Catalog #**M402A
- Illumina FWD and REV barcode primers
- Gel electrophoresis reagents and equipment.
- 1X TE Low EDTA Buffer
- PromethION 2 solo and device specific flow cells
- Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #**SQK-LSK114

PCR2 Forward Primers

	A	B	C
	Well Position	**Name**	**Sequence**
	A01	hybrid_dual_fw_001	AATGATACGGCGACCACCGAGATCTACACGTTAAGACACACTCTTTCCCTACACGACTCGCCAAGCTGA
	A02	hybrid_dual_fw_002	AATGATACGGCGACCACCGAGATCTACACTCTAAGTTACACTCTTTCCCTACACGACTCGCCAAGCTGA
	A03	hybrid_dual_fw_003	AATGATACGGCGACCACCGAGATCTACACTGTACATTACACTCTTTCCCTACACGACTCGCCAAGCTGA
	A04	hybrid_dual_fw_004	AATGATACGGCGACCACCGAGATC

	A	B	C
			TACACAATA CCGCACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	A05	hybrid_dual_ fw_005	AATGATAC GGCGACCA CCGAGATC TACACTGT CTATGACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	A06	hybrid_dual_ fw_006	AATGATAC GGCGACCA CCGAGATC TACACTCA CTGTTACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	A07	hybrid_dual_ fw_007	AATGATAC GGCGACCA CCGAGATC TACACTTG TATTCACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	A08	hybrid_dual_ fw_008	AATGATAC GGCGACCA CCGAGATC TACACAGA CTAACACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	A09	hybrid_dual_ fw_009	AATGATAC GGCGACCA CCGAGATC TACACATTG TCAAACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	A10	hybrid_dual_ fw_010	AATGATAC GGCGACCA CCGAGATC

	A	B	C
			TACACGTT CTACAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	A11	hybrid_dual_fw_011	AATGATAC GGCGACCA CCGAGATC TACACTTC ATTCGACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	A12	hybrid_dual_fw_012	AATGATAC GGCGACCA CCGAGATC TACACAAG CGTCAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	B01	hybrid_dual_fw_013	AATGATAC GGCGACCA CCGAGATC TACACCGT AGGATACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	B02	hybrid_dual_fw_014	AATGATAC GGCGACCA CCGAGATC TACACAGT TAGCGACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	B03	hybrid_dual_fw_015	AATGATAC GGCGACCA CCGAGATC TACACGAA CGTCTACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	B04	hybrid_dual_fw_016	AATGATAC GGCGACCA CCGAGATC

	A	B	C
			TACACAGA ACACTACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	B05	hybrid_dual_fw_017	AATGATAC GGCGACCA CCGAGATC TACACTGT TGGCAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	B06	hybrid_dual_fw_018	AATGATAC GGCGACCA CCGAGATC TACACGGC TAATAACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	B07	hybrid_dual_fw_019	AATGATAC GGCGACCA CCGAGATC TACACGCT AGAATACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	B08	hybrid_dual_fw_020	AATGATAC GGCGACCA CCGAGATC TACACTGTA CTAAACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	B09	hybrid_dual_fw_021	AATGATAC GGCGACCA CCGAGATC TACACAGT TACACACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	B10	hybrid_dual_fw_022	AATGATAC GGCGACCA CCGAGATC

	A	B	C
			TACACGTA CACGAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	B11	hybrid_dual_ fw_023	AATGATAC GGCGACCA CCGAGATC TACACACT GTAAGACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	B12	hybrid_dual_ fw_024	AATGATAC GGCGACCA CCGAGATC TACACGAT CATGAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	C01	hybrid_dual_ fw_025	AATGATAC GGCGACCA CCGAGATC TACACTGC TAACGACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	C02	hybrid_dual_ fw_026	AATGATAC GGCGACCA CCGAGATC TACACTAAT GCTCACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	C03	hybrid_dual_ fw_027	AATGATAC GGCGACCA CCGAGATC TACACCTT GTTGAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	C04	hybrid_dual_ fw_028	AATGATAC GGCGACCA CCGAGATC

	A	B	C
			TACACGATA CAAGACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	C05	hybrid_dual_ fw_029	AATGATAC GGCGACCA CCGAGATC TACACATAC AGCGACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	C06	hybrid_dual_ fw_030	AATGATAC GGCGACCA CCGAGATC TACACCTG GATAGACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	C07	hybrid_dual_ fw_031	AATGATAC GGCGACCA CCGAGATC TACACTCAT CAGCACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	C08	hybrid_dual_ fw_032	AATGATAC GGCGACCA CCGAGATC TACACTTAT ACCGACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	C09	hybrid_dual_ fw_033	AATGATAC GGCGACCA CCGAGATC TACACGCT GATTCACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	C10	hybrid_dual_ fw_034	AATGATAC GGCGACCA CCGAGATC

	A	B	C
			TACACATAA GGCCACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	C11	hybrid_dual_ fw_035	AATGATAC GGCGACCA CCGAGATC TACACAAG ACTAGACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	C12	hybrid_dual_ fw_036	AATGATAC GGCGACCA CCGAGATC TACACTAG CAACAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	D01	hybrid_dual_ fw_037	AATGATAC GGCGACCA CCGAGATC TACACCAA GTAATACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	D02	hybrid_dual_ fw_038	AATGATAC GGCGACCA CCGAGATC TACACTCAT GATGACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	D03	hybrid_dual_ fw_039	AATGATAC GGCGACCA CCGAGATC TACACTAG CTCTTACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	D04	hybrid_dual_ fw_040	AATGATAC GGCGACCA CCGAGATC

	A	B	C
			TACACACT ACATGACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	D05	hybrid_dual_fw_041	AATGATAC GGCGACCA CCGAGATC TACACTGT GCCATACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	D06	hybrid_dual_fw_042	AATGATAC GGCGACCA CCGAGATC TACACGAC TTCCAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	D07	hybrid_dual_fw_043	AATGATAC GGCGACCA CCGAGATC TACACTATT CTCGACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	D08	hybrid_dual_fw_044	AATGATAC GGCGACCA CCGAGATC TACACCAC TAGATACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	D09	hybrid_dual_fw_045	AATGATAC GGCGACCA CCGAGATC TACACCTT CGCATACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	D10	hybrid_dual_fw_046	AATGATAC GGCGACCA CCGAGATC

	A	B	C
			TACACCTA GCGATACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	D11	hybrid_dual_fw_047	AATGATAC GGCGACCA CCGAGATC TACACTAC GTTAAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	D12	hybrid_dual_fw_048	AATGATAC GGCGACCA CCGAGATC TACACAAG TAGTCACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	E01	hybrid_dual_fw_049	AATGATAC GGCGACCA CCGAGATC TACACGAC GTATCACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	E02	hybrid_dual_fw_050	AATGATAC GGCGACCA CCGAGATC TACACACG TTCAGACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	E03	hybrid_dual_fw_051	AATGATAC GGCGACCA CCGAGATC TACACAGC TATATACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	E04	hybrid_dual_fw_052	AATGATAC GGCGACCA CCGAGATC

	A	B	C
			TACACAGT GCGAAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	E05	hybrid_dual_fw_053	AATGATAC GGCGACCA CCGAGATC TACACCAA TGGTAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	E06	hybrid_dual_fw_054	AATGATAC GGCGACCA CCGAGATC TACACATTC CGATACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	E07	hybrid_dual_fw_055	AATGATAC GGCGACCA CCGAGATC TACACAGC TGTTGACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	E08	hybrid_dual_fw_056	AATGATAC GGCGACCA CCGAGATC TACACTTC GACTCACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	E09	hybrid_dual_fw_057	AATGATAC GGCGACCA CCGAGATC TACACCGA ATGGTACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	E10	hybrid_dual_fw_058	AATGATAC GGCGACCA CCGAGATC

	A	B	C
			TACACTAGT GTACACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	E11	hybrid_dual_ fw_059	AATGATAC GGCGACCA CCGAGATC TACACACG CATGAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	E12	hybrid_dual_ fw_060	AATGATAC GGCGACCA CCGAGATC TACACTTC GTACAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	F01	hybrid_dual_ fw_061	AATGATAC GGCGACCA CCGAGATC TACACTTC TGCTAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	F02	hybrid_dual_ fw_062	AATGATAC GGCGACCA CCGAGATC TACACTGG AAGCAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	F03	hybrid_dual_ fw_063	AATGATAC GGCGACCA CCGAGATC TACACGAT GCTCAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	F04	hybrid_dual_ fw_064	AATGATAC GGCGACCA CCGAGATC

	A	B	C
			TACACAGC CTGAAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	F05	hybrid_dual_fw_065	AATGATAC GGCGACCA CCGAGATC TACACCAC CAGTAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	F06	hybrid_dual_fw_066	AATGATAC GGCGACCA CCGAGATC TACACGTTA CACCACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	F07	hybrid_dual_fw_067	AATGATAC GGCGACCA CCGAGATC TACACGCG TTAGTACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	F08	hybrid_dual_fw_068	AATGATAC GGCGACCA CCGAGATC TACACTGA CGTTCACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	F09	hybrid_dual_fw_069	AATGATAC GGCGACCA CCGAGATC TACACTAG GCGTAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	F10	hybrid_dual_fw_070	AATGATAC GGCGACCA CCGAGATC

	A	B	C
			TACACAAG TGAGCACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	F11	hybrid_dual_fw_071	AATGATAC GGCGACCA CCGAGATC TACACCCG GAATAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	F12	hybrid_dual_fw_072	AATGATAC GGCGACCA CCGAGATC TACACAATA GGCAACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	G01	hybrid_dual_fw_073	AATGATAC GGCGACCA CCGAGATC TACACTTC AGGTCACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	G02	hybrid_dual_fw_074	AATGATAC GGCGACCA CCGAGATC TACACTAAT CGCTACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	G03	hybrid_dual_fw_075	AATGATAC GGCGACCA CCGAGATC TACACCTT AGGTGACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	G04	hybrid_dual_fw_076	AATGATAC GGCGACCA CCGAGATC

	A	B	C
			TACACTTG ATCTCACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	G05	hybrid_dual_fw_077	AATGATAC GGCGACCA CCGAGATC TACACTCT TACTGACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	G06	hybrid_dual_fw_078	AATGATAC GGCGACCA CCGAGATC TACACGAG TACACACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	G07	hybrid_dual_fw_079	AATGATAC GGCGACCA CCGAGATC TACACACG TTACAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	G08	hybrid_dual_fw_080	AATGATAC GGCGACCA CCGAGATC TACACTGG TAAGCACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	G09	hybrid_dual_fw_081	AATGATAC GGCGACCA CCGAGATC TACACGATA GCACACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	G10	hybrid_dual_fw_082	AATGATAC GGCGACCA CCGAGATC

	A	B	C
			TACACGAC TTAGGACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	G11	hybrid_dual_fw_083	AATGATAC GGCGACCA CCGAGATC TACACAGT CGTTAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	G12	hybrid_dual_fw_084	AATGATAC GGCGACCA CCGAGATC TACACCGT GATTAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	H01	hybrid_dual_fw_085	AATGATAC GGCGACCA CCGAGATC TACACTCA GTAGAACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	H02	hybrid_dual_fw_086	AATGATAC GGCGACCA CCGAGATC TACACATGT GTCTACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	H03	hybrid_dual_fw_087	AATGATAC GGCGACCA CCGAGATC TACACCAG AATATACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	H04	hybrid_dual_fw_088	AATGATAC GGCGACCA CCGAGATC

	A	B	C
			TACACTAA GTCTGACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	H05	hybrid_dual_fw_089	AATGATAC GGCGACCA CCGAGATC TACACATC GACATACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	H06	hybrid_dual_fw_090	AATGATAC GGCGACCA CCGAGATC TACACACA GCTGTACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	H07	hybrid_dual_fw_091	AATGATAC GGCGACCA CCGAGATC TACACATCA CAAGACAC TCTTTCCC TACACGAC TCGCCAAG CTGA
	H08	hybrid_dual_fw_092	AATGATAC GGCGACCA CCGAGATC TACACGTC GAATTACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	H09	hybrid_dual_fw_093	AATGATAC GGCGACCA CCGAGATC TACACTGC CGATTACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	H10	hybrid_dual_fw_094	AATGATAC GGCGACCA CCGAGATC

	A	B	C
			TACACACT AATGCACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	H11	hybrid_dual_fw_095	AATGATAC GGCGACCA CCGAGATC TACACCTA GTAGGACA CTCTTTCC CTACACGA CTCGCCAA GCTGA
	H12	hybrid_dual_fw_096	AATGATAC GGCGACCA CCGAGATC TACACTTAA GCCAACAC TCTTTCCC TACACGAC TCGCCAAG CTGA

PCR2 Reverse Primers

	Well Position	Name	Sequence
	A01	hybrid_dual_rev_001	CAAGCAGAAGACGGCATACGAGATGTTAAGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	A02	hybrid_dual_rev_002	CAAGCAGAAGACGGCATACGAGATTCTAAGTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	A03	hybrid_dual_rev_003	CAAGCAGAAGACGGCATACGAGATTGTACATTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	A04	hybrid_dual_rev_004	CAAGCAGAAGACGGCATACGAGATAATACCGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	A05	hybrid_dual_rev_005	CAAGCAGAAGACGGCATACGAGATTGTCTATGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	A06	hybrid_dual_rev_006	CAAGCAGAAGACGGCATACGAGATTCACTGTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	A07	hybrid_dual_rev_007	CAAGCAGAAGACGGCATACGAGATTTGTATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

	A08	hybrid_dual_rev_008	CAAGCAGAAGACGGCATACGAGATAGACTAACGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	A09	hybrid_dual_rev_009	CAAGCAGAAGACGGCATACGAGATATTGTCAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	A10	hybrid_dual_rev_010	CAAGCAGAAGACGGCATACGAGATGTTCTACAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	A11	hybrid_dual_rev_011	CAAGCAGAAGACGGCATACGAGATTTCATTCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	A12	hybrid_dual_rev_012	CAAGCAGAAGACGGCATACGAGATAAGCGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	B01	hybrid_dual_rev_013	CAAGCAGAAGACGGCATACGAGATCGTAGGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	B02	hybrid_dual_rev_014	CAAGCAGAAGACGGCATACGAGATAGTTAGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	B03	hybrid_dual_rev_015	CAAGCAGAAGACGGCATACGAGATGAACGTCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	B04	hybrid_dual_rev_016	CAAGCAGAAGACGGCATACGAGATAGAACACTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	B05	hybrid_dual_rev_017	CAAGCAGAAGACGGCATACGAGATTGTTGGCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	B06	hybrid_dual_rev_018	CAAGCAGAAGACGGCATACGAGATGGCTAATAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	B07	hybrid_dual_rev_019	CAAGCAGAAGACGGCATACGAGATGCTAGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	B08	hybrid_dual_rev_020	CAAGCAGAAGACGGCATACGAGATTGTACTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	B09	hybrid_dual_rev_021	CAAGCAGAAGACGGCATACGAGATAGTTACACGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	B10	hybrid_dual_rev_022	CAAGCAGAAGACGGCATACGAGATGTACACGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	B11	hybrid_dual_rev_023	CAAGCAGAAGACGGCATACGAGATACTGTAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	B12	hybrid_dual_rev_024	CAAGCAGAAGACGGCATACGAGATGATCATGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	C01	hybrid_dual_rev_025	CAAGCAGAAGACGGCATACGAGATTGCTAACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	C02	hybrid_dual_rev_026	CAAGCAGAAGACGGCATACGAGATTAATGCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

	C03	hybrid_dual_rev_027	CAAGCAGAAGACGGCATACGAGATCTTGTTGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	C04	hybrid_dual_rev_028	CAAGCAGAAGACGGCATACGAGATGATACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	C05	hybrid_dual_rev_029	CAAGCAGAAGACGGCATACGAGATATACAGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	C06	hybrid_dual_rev_030	CAAGCAGAAGACGGCATACGAGATCTGGATAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	C07	hybrid_dual_rev_031	CAAGCAGAAGACGGCATACGAGATTCATCAGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	C08	hybrid_dual_rev_032	CAAGCAGAAGACGGCATACGAGATTTATACCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	C09	hybrid_dual_rev_033	CAAGCAGAAGACGGCATACGAGATGCTGATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	C10	hybrid_dual_rev_034	CAAGCAGAAGACGGCATACGAGATATAAGGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	C11	hybrid_dual_rev_035	CAAGCAGAAGACGGCATACGAGATAAGACTAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	C12	hybrid_dual_rev_036	CAAGCAGAAGACGGCATACGAGATTAGCAACAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	D01	hybrid_dual_rev_037	CAAGCAGAAGACGGCATACGAGATCAAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	D02	hybrid_dual_rev_038	CAAGCAGAAGACGGCATACGAGATTCATGATGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	D03	hybrid_dual_rev_039	CAAGCAGAAGACGGCATACGAGATTAGCTCTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	D04	hybrid_dual_rev_040	CAAGCAGAAGACGGCATACGAGATACTACATGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	D05	hybrid_dual_rev_041	CAAGCAGAAGACGGCATACGAGATTGTGCCATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	D06	hybrid_dual_rev_042	CAAGCAGAAGACGGCATACGAGATGACTTCCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	D07	hybrid_dual_rev_043	CAAGCAGAAGACGGCATACGAGATTATTCTCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	D08	hybrid_dual_rev_044	CAAGCAGAAGACGGCATACGAGATCACTAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	D09	hybrid_dual_rev_045	CAAGCAGAAGACGGCATACGAGATCTTCGCATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

	D10	hybrid_dual_rev_046	CAAGCAGAAGACGGCATACGAGATCTAGCGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	D11	hybrid_dual_rev_047	CAAGCAGAAGACGGCATACGAGATTACGTTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	D12	hybrid_dual_rev_048	CAAGCAGAAGACGGCATACGAGATAAGTAGTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	E01	hybrid_dual_rev_049	CAAGCAGAAGACGGCATACGAGATGACGTATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	E02	hybrid_dual_rev_050	CAAGCAGAAGACGGCATACGAGATACGTTCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	E03	hybrid_dual_rev_051	CAAGCAGAAGACGGCATACGAGATAGCTATATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	E04	hybrid_dual_rev_052	CAAGCAGAAGACGGCATACGAGATAGTGCGAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	E05	hybrid_dual_rev_053	CAAGCAGAAGACGGCATACGAGATCAATGGTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	E06	hybrid_dual_rev_054	CAAGCAGAAGACGGCATACGAGATATTCCGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	E07	hybrid_dual_rev_055	CAAGCAGAAGACGGCATACGAGATAGCTGTTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	E08	hybrid_dual_rev_056	CAAGCAGAAGACGGCATACGAGATTTCGACTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	E09	hybrid_dual_rev_057	CAAGCAGAAGACGGCATACGAGATCGAATGGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	E10	hybrid_dual_rev_058	CAAGCAGAAGACGGCATACGAGATTAGTGTACGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	E11	hybrid_dual_rev_059	CAAGCAGAAGACGGCATACGAGATACGCATGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	E12	hybrid_dual_rev_060	CAAGCAGAAGACGGCATACGAGATTTCGTACAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	F01	hybrid_dual_rev_061	CAAGCAGAAGACGGCATACGAGATTTCTGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	F02	hybrid_dual_rev_062	CAAGCAGAAGACGGCATACGAGATTGGAAGCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	F03	hybrid_dual_rev_063	CAAGCAGAAGACGGCATACGAGATGATGCTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	F04	hybrid_dual_rev_064	CAAGCAGAAGACGGCATACGAGATAGCCTGAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

	F05	hybrid_dual_rev_065	CAAGCAGAAGACGGCATACGAGATCACCAGTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	F06	hybrid_dual_rev_066	CAAGCAGAAGACGGCATACGAGATGTTACACCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	F07	hybrid_dual_rev_067	CAAGCAGAAGACGGCATACGAGATGCGTTAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	F08	hybrid_dual_rev_068	CAAGCAGAAGACGGCATACGAGATTGACGTTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	F09	hybrid_dual_rev_069	CAAGCAGAAGACGGCATACGAGATTAGGCGTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	F10	hybrid_dual_rev_070	CAAGCAGAAGACGGCATACGAGATAAGTGAGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	F11	hybrid_dual_rev_071	CAAGCAGAAGACGGCATACGAGATCCGGAATAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	F12	hybrid_dual_rev_072	CAAGCAGAAGACGGCATACGAGATAATAGGCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	G01	hybrid_dual_rev_073	CAAGCAGAAGACGGCATACGAGATTTCAGGTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	G02	hybrid_dual_rev_074	CAAGCAGAAGACGGCATACGAGATTAATCGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	G03	hybrid_dual_rev_075	CAAGCAGAAGACGGCATACGAGATCTTAGGTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	G04	hybrid_dual_rev_076	CAAGCAGAAGACGGCATACGAGATTTGATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	G05	hybrid_dual_rev_077	CAAGCAGAAGACGGCATACGAGATTCTTACTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	G06	hybrid_dual_rev_078	CAAGCAGAAGACGGCATACGAGATGAGTACACGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	G07	hybrid_dual_rev_079	CAAGCAGAAGACGGCATACGAGATACGTTACAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	G08	hybrid_dual_rev_080	CAAGCAGAAGACGGCATACGAGATTGGTAAGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	G09	hybrid_dual_rev_081	CAAGCAGAAGACGGCATACGAGATGATAGCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	G10	hybrid_dual_rev_082	CAAGCAGAAGACGGCATACGAGATGACTTAGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	G11	hybrid_dual_rev_083	CAAGCAGAAGACGGCATACGAGATAGTCGTTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

	G12	hybrid_dual_rev_084	CAAGCAGAAGACGGCATACGAGATCGTGATTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	H01	hybrid_dual_rev_085	CAAGCAGAAGACGGCATACGAGATTCAGTAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	H02	hybrid_dual_rev_086	CAAGCAGAAGACGGCATACGAGATATGTGTCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	H03	hybrid_dual_rev_087	CAAGCAGAAGACGGCATACGAGATCAGAATATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	H04	hybrid_dual_rev_088	CAAGCAGAAGACGGCATACGAGATTAAGTCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	H05	hybrid_dual_rev_089	CAAGCAGAAGACGGCATACGAGATATCGACATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	H06	hybrid_dual_rev_090	CAAGCAGAAGACGGCATACGAGATACAGCTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	H07	hybrid_dual_rev_091	CAAGCAGAAGACGGCATACGAGATATCACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	H08	hybrid_dual_rev_092	CAAGCAGAAGACGGCATACGAGATGTCGAATTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	H09	hybrid_dual_rev_093	CAAGCAGAAGACGGCATACGAGATTGCCGATTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	H10	hybrid_dual_rev_094	CAAGCAGAAGACGGCATACGAGATACTAATGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	H11	hybrid_dual_rev_095	CAAGCAGAAGACGGCATACGAGATCTAGTAGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
	H12	hybrid_dual_rev_096	CAAGCAGAAGACGGCATACGAGATTTAAGCCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

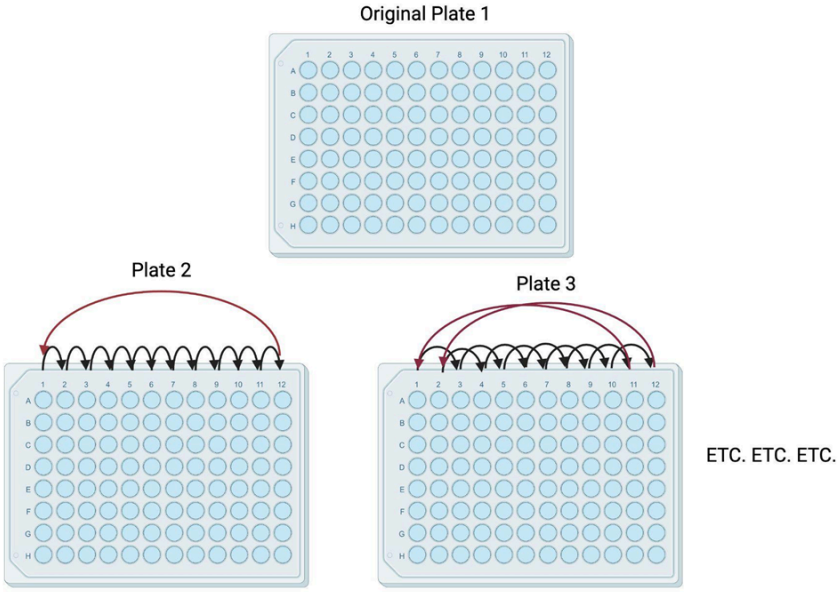
Preparation of PCR2 Primer plates

1 To allow for high levels of combinations of indexing primers, we generate multiple forward primer plates (12) from a single 96-well plate.

2 By using these 12 plates in combination with a single 96-well plate of reverse primers, we can index 1,152 samples.

3 Additional primers can be designed to increase this multiplexing. Here we present one plate of forward and one plate of reverse primers to allow for this level of combination.

4 To generate forward primer plates, we shift the column to the right one column when replating to a new plate (e.g. column 1 becomes column 2 and column 12 becomes column 1). See the figure below. Be sure to uniquely label the plate so you know which primers are used in library preparation.



Note

We do NOT do this for reverse primers.

Generate Primers mix

5 Spin lyophilized Primer.

6 Resuspend in MGW using the provided volume for a [M] 100 micromolar (µM) stock.

7 Vortex and spin down briefly.

8 Let the mixture sit for about 00:10:00 for complete dissolution.

9 Prepare the stock primer pool ([M] 100 micromolar (µM)) by mixing 1 volume of sp-rtss-repeat_v4_F_MIP with 1 volume of csp-rtss-repeat_v4_R_MIP.

10 Dilute primer pool with MGW to obtain [M] 20 micromolar (µM) working solution: 1 volume of stock + 4 volumes of MGW.

PCR1

2m

11 Pre-set the thermocycler before starting master mix preparation using the programme below.

	A	B	C	D
	Step	**Temperature (°C)**	**Time (minute)**	**Number of cycles**
	Step1 (preheat)	95	2	1
	Step2 (Activation)	95	0.5	35
	Step3 (Denaturation)	94	0.5	
	Step4 (Annealing)	59	1	

	A	B	C	D
	Step5 (final Extension)	72	10	1
	Step6	4	hold	NA

12 All sample plates and controls are to be kept on cold blocks - switch out cold blocks if they are getting warm.

13 All reagents are to be kept On ice and the tube used to prepare the master mix should also remain On ice .

14 Centrifuge sample plates at 4000 rpm, 00:01:00 . 1m

15 Prepare the master mix using the template (here 96 reactions) below and adjust it to the number of reactions.

These volumes could be halved but not tested at that volume.

	A	B	C
	Master mix	**x1**	**x96* 1.1**
	Gotaq	25	2,640
	Molecular grade water	19	2,6006.4
	Primer pool (P2_Px1Block3_v2_F and P2_Px1Frag3_v8_R)	1	105.6
	Template DNA	5	

16 Divide the total master mix volume by 12 and aliquot the designated volume of into 12 tube strip on cold block to allow for multichannel pipetting.

17 Using a multichannel, pipette 16 µL of master mix into each well of 96 well plate..

18 Using a multichannel, plate 4 µL of sample into each well on the PCR1 plate.

- Add at least one positive control (lab strain, 4 µL) and one PCR1 negative control (MGW, 4 µL).

19 Centrifuge the plate at 4000 rpm, 00:01:00 . 1m

20 Place the plate in the thermocycler and start running the pre-set PCR program.

PCR2 or barcoding PCR 3m

21 Once the first PCR is complete, pre-set the thermocycler before starting the master mix preparation for PCR2 using the program below.

	A	B	C	D
	Step	**Temperature (ºC)**	**Time (minute)**	**Number of cycles**
	Step1	98	hold	NA
	Step2	98	0:30	NA
	Step3	95	0:10	x10
	Step4	62	8:0	x10
	Step5	72	0:20	x10
	Step6	72	1:0	NA
	Step7	4	hold	NA

22 Prepare the master mix for PCR2 using the template (here 96 reactions) below and adjust it to the number of reactions.

	A	B	C
	Master mix	**x1**	**x96* 1.1**
	Gotaq	10	1056
	Molecular grade water	6	633.6

23 Divide the total master mix volume by 12 and aliquot the designated volume (140.8 µL for 96 sample run) into 12 tube strip on cold block to allow for multichannel pipetting.

24 Using a multichannel, pipette 16 µL of master mix into each well.

25 Spin down the forward and reverse PCR2 primer plates needed for the experiment at 4000 rpm, 00:01:00 and add 1 µL of forward primer and 1 µL of reverse primer into each well using the multichannel.

1m

- On your sample sheet, please denote the name of the forward and reverse primer plate used and the well of the sample to be sure you know the correct index for the sample you are genotyping.

26 In the post-PCR lab, add 2 µL of the PCR1 product to the master mix using a multichannel.

27 **Optional:** shake the plate gently on a shaker for 00:01:00 to mix the master mix added to the samples.

1m

28 Centrifuge the plate at 4000 rpm, 00:01:00 .

1m

29 Place the plate in the thermocycler and start running the pre-set PCR program.

Check a random set of 10 wells per plate for amplification by gel electrophoresis based on your lab protocol (including the NTC and positive control).

PCR Product Pooling and Cleanup

10m

30 Pool 5 µL of PCR2 product into a single Eppendorf tube. If the pool is very large, 3 µL is sufficient.

31 Bring Ampure Beads to Room temperature. This can also be done with Spherotech beads (DIY).

32 Add an equal volume of beads to the pool (1X bead clean). Let it incubate for at least 00:10:00 at Room temperature. For a higher yield, place the tube on a Hula Mixer or invert it in your hand.

33 Place the pool on a magnet and wait until the beads have pelletted on the magnet and the solution is clear. Prepare enough fresh 80% ethanol to cover the beads for 2 washes.

34 Remove the supernatant and reserve it in another tube.

35 Wash with 80% ethanol for at least 00:00:30. Discard the ethanol.

36 Repeat the previous step for a second wash.

37 Remove the ethanol and let the beads dry for around 00:01:00 (do not let the beads dry until cracking).

38 Elute in 51 µL of 1X low EDTA TE Buffer. If the pool is very large, use your discretion and elute in a higher volume (we eluted in 150 µL with 6 96-well plates of sample). Incubate at Room temperature for 00:10:00.

10m

39 Pellet the beads on a magnet and remove the elution into another tube.

40 Quantify the elution with a Qubit fluorometer.

Nanopore Library preparation

41 We use the SQK-LSK114 Kit (Ligation Sequencing) and have adapted this protocol from the Ligation Sequencing Amplicons V14 protocol from Nanopore. We use the P2 sequencing machine, but this protocol could be adapted for Minion.

Nanopore Library preparation - End Prep

26m 30s

42 Transfer 200 fmol of cleaned DNA into a PCR tube or plate. Calculation for fmol can be done on this website: **https://www.promega.com/resources/tools/biomath/** (1 pmol = 1,000 fmol). Adjust the volume to 50 µL with nuclease-free water.

43 Add the following to a PCR tube:

	A	B
	Reagent	**Volume**
	DNA Template	50 µL
	Ultra II End Prep Reaction Buffer	7 µL
	Ultra II End Prep Enzyme Mix	3 µL
	Total	**60µL**

44 Mix thoroughly by pipetting.

45 Incubate at 20 °C for 00:05:00 and 65 °C for 00:05:00 .

10m

46 Spin down and transfer the sample to a clean 1.5ml Eppendorf tube.

47 Bring beads to Room temperature and add 60 µL to the DNA sample.

48 Incubate for 00:10:00 at Room temperature. For increased yield, place the tube on a Hula Mixer or invert in your hand. Meanwhile, prepare 1 mL of 80% ethanol. 10m

49 Pellet on a magnet and remove the supernatant - keep the supernatant for extra security.

50 Wash with 500 µL of 80% ethanol. Remove after 00:00:30. 30s

51 Repeat the previous step for a total of 2 washes.

51.1 Wash with 500 µL of 80% ethanol. Remove after 00:00:30 (1/2). 30s

51.2 Wash with 500 µL of 80% ethanol. Remove after 00:00:30 (2/2). 30s

52 Let the beads dry for around 00:01:00. Do not let the beads crack on the side of the tube.

53 Elute in 61 µL of nuclease-free water. Let it incubate at Room temperature for 00:05:00. 5m

54 Pellet on the magnet and remove the elution.

55 Quantify the End-prepped DNA with a qubit fluorometer. This library can be stored at 4 °C for short-term storage, or at -20 °C or -80 °C for long-term storage.

Nanopore Library preparation - Adapter Ligation 21m 30s

56 Thaw the Short Fragment Buffer (SFB) and the Elution Buffer (EB) at Room temperature. Place the Ligation Adapter (LA), Ligation Buffer (LNB), and Quick T4 Ligase On ice.

57 Mix the following in a 1.5 ml Eppendorf tube, mixing 10-20 times after each addition:

	A	B
	Reagent	**Volume**
	End-prepped DNA	60 µL
	Ligation Buffer (LNB)	25 µL
	NEBNext Quick T4 Ligase	10 µL
	Ligation Adapter (LA)	5 µL
	Total	**100 µL**

58 Mix well by pipetting. Incubate at Room temperature for at least 00:10:00. A longer incubation of 00:20:00 will increase the amount of ligated adapters.

59 Bring the beads to Room temperature and resuspend.

60 Add 60 µL of beads and mix by inverting. Incubate for 00:10:00 at Room temperature. For higher yield, invert in your hand or place on a Hula mixer

10m

61 Pellet on a magnet and remove the supernatant.

62 Wash with 250 µL of the Short Fragment buffer. Remove after around 00:00:30.

30s

63 Repeat the previous step for a total of 2 washes.

63.1 Wash with 250 µL of the Short Fragment buffer. Remove after around 00:00:30 (1/2). 30s

63.2 Wash with 250 µL of the Short Fragment buffer. Remove after around 00:00:30 (2/2). 30s

64 Allow beads to dry on the magnet, and pipette out any residual buffer. Do not let beads dry to the point of cracking.

65 Elute in 25 µL of the Elution Buffer (EB). Incubate for 00:10:00 at Room temperature . 10m

66 Pellet on a magnet and pipette the elution into a tube (ideally a screw cap tube for best storage).

67 Quantify the library with a qubit fluorometer. Be sure that you have at least 120 fmol of DNA, which is around 25 ng of DNA for a 4CAST library. This library can be stored at 4 °C for short-term storage, or at -20 °C or -80 °C for long-term storage.

Nanopore sequencing - Loading the Flow Cell 20m

68 Take the Promethion Flow Cell you plan to use out of the 4 °C refrigerator at least 00:20:00 before use. It is highly recommended to do a flow cell check before any sequencing.

69 Determine 100 - 120 fmols of library and add to an Eppendorf tube (Nanopore recommends 20 fmol for sequencing; however, this leads to underloading of the flow cell in most cases. If you have less than 100 fmols, add the entire library). 25 ng of 4CAST Library is around 120 fmols.

70 Bring up the 100 - 120 fmols of library to 32 µL with the elution buffer (EB).

71 After 00:20:00 at Room temperature, gently insert the flow cell into one of the ports of the P2. The lights will turn blue if inserted correctly, and red if inserted incorrectly.

72 In a 1.5 Eppendorf tube, mix the following to create the flow cell priming mix:

	A	B
	Reagent	**Volume**
	Flow Cell Flush (FCF)	1170 µL
	Flow Cell Tether (FCT)	30 µL
	Total	**1200 µL**

73 Set a P1000 pipette to 200. Open the inlet port and place the tip inside the port. Turn the dial until it shows 220-230. A small amount of buffer should enter the tip. Discard the tip and buffer.

74 Load 500 µL of the flow cell priming mix into the inlet port. Load by slowly twisting the dial in the counterclockwise direction. Wait 00:05:00. 5m

75 While the priming mix is incubating, prepare the library:

	A	B
	Reagent	**Volume**
	Sequencing Buffer (SB)	100 µL
	Library Beads (LIB)	68 µL

	A	B
	DNA Library	32 µL
	Total	**200 µL**

76 After the 00:05:00 incubation, add another 500 µL of flow cell priming mix to the inlet port. 5m

77 Immediately add the 200 µL of the library slowly into the inlet port.

78 Close the inlet port and wait for the library to incubate for 00:10:00 before starting any sequencing. 10m

Nanopore sequencing - Flow cell wash 1h

79 A flow cell wash should be done as soon as possible after the sequencing run is complete.

80 Place the Wash Mix (WMX) On ice, and thaw the Wash Diluent (DIL) at Room temperature.

81 Vortex the Wash Diluent thoroughly.

82 Make the Flow Cell Wash Mix by adding the following to a 1.5ml Eppendorf tube:

	A	B
	Reagent	**Volume**
	Wash Diluent (DIL)	398 µL
	Wash Mix (WMX)	2 µL

83 Pipette to mix and place On ice .

84 Stop the sequencing run on Minknow, and leave the flow cell in the port.

85 Make sure the inlet port is closed, and remove waste from port 2 or port 3.

86 Open the inlet port and set the P1000 to 200. Remove the air bubble by turning the wheel until it reaches 220-230, and a small volume of buffer enters the pipette tip.

87 Load 400 µL of the Flow Cell Wash Mix into the inlet port. Incubate for 01:00:00 . 1h

88 After 01:00:00 , remove the waste again from ports 2 or 3. Make sure the inlet port is closed.

89 Remove the air bubble if it is present after removing the waste. Then add 500 µL of Storage Buffer (S). The flow cell can now be placed back into a 4 °C refrigerator for long-term storage. Incubate the flow cell at least Overnight before doing another flow cell check.