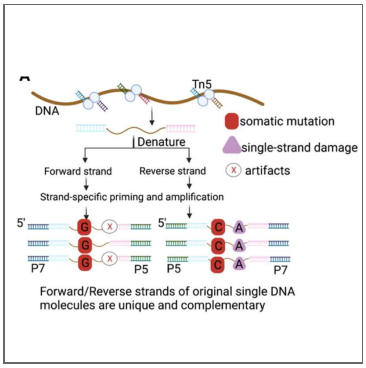

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MANUSCRIPT CITATION:

Tn5-Duplex-Sequencing (Tn5-Duplex-Seq) for low-input single-molecule variant detection

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Nazia Hilal

CITATION

Xing D, Tan L, Chang CH, Li H, Xie XS (2021). Accurate SNV detection in single cells by transposon-based whole-genome amplification of complementary strands..
LINK
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We use this protocol and it's working

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Keywords: Duplex-sequencing technology, Tn5-duplex-sequencing, Somatic mutations, single nucleotide variants (SNVs), variation calling, copy number, variant allele frequency (VAF) analysis

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ABSTRACT

DNA mutations are the inevitable consequences of errors that arise during replication-repair of DNA damage as well as aging and disease progression. Because of their random and infrequent occurrence, quantification, and characterization of DNA mutations in the genome of somatic cells have been difficult. These mutations in DNA drive genetic diversity, alter gene function, define evolutionary trajectories, and provide targets for precision medicine and diagnostics. It is crucial to detect mutations across a wide range of abundance, i.e., variant allele frequency (VAF). Detecting low-abundance mutations (e.g., <0.1–1% VAF or in individual cells) is important for understanding human embryonic development, somatic mosaicism, and clonal hematopoiesis and uncovering pathogenic variants. Altogether somatic mutations provide important and unique insights into the biology of complex diseases. *To decipher the causal inference, we must build robust genetic maps of somatic evolution in health and disease.* The recent advent of duplex consensus sequencing has heralded a new generation of accuracy. However, multiple techniques focus on targeted areas of the genome (Twin Strand Biosciences) or are limited to restriction sites (Nanoseq), limiting their application to comprehensive somatic variant characterization. Furthermore, fragmentation of the genome and standard A-tailing and ligation creates errors (BotseqS, CODEC). Ligation of duplex strands for efficient sequencing has proven promising, though in practice requires complex molecular structures (Pro-Seq, CODEC) which have been observed to frequently result in incorrectly paired duplexes (CODEC). To enable comprehensive variant detection by next-generation DNA sequencing, we propose an innovative, accessible, and highly accurate Tn5 transposase-based duplex-sequencing technology (**Tn5-duplex-seq**) where complementary strands of DNA could be labeled at the molecular level in a single-tube reaction; thus, identifying single nucleotide variants (SNVs) from **single-molecules of DNA regardless of starting from single cells or pooled cell/DNA input.** The conceptual basis of the protocol comes from META-CS (Xing et al.2021), a Tn5 based aproach optimized for single-cell whole genome amplification. We find that modifications of this approach to include flexible input and the sequencing strategy to optimize cost per variant detection enables great flexibility for all low-input applications.
Tn5-duplex-seq approach offers several benefits over other duplex approaches including.

(1) preservation of original template molecules by utilizing 16 unique sequences (Compared to the loss of 50% of
molecules due to intramolecular symmetry during TN5-based Nextera library preparation)
(2) accuracy by eliminating the requirement for A-tailing
(3) efficiency of duplex capture through specifying input
(4) accessibility by using standard reagents and oligonucleotide preparations
(5) distinction between double-stranded SNVs and single-stranded lesions.

Our method enables library preparation for short-read sequencing. Downstream analysis enables accurate and high-throughput SNV/indel and copy number analysis.

GUIDELINES

Optimization of proteinase K concentration for Step 1:
As different cell types may vary in the degree of chromatin condensation and material, we recommend titration of proteinase K at 0.5X, 1X, 5X, 10X our recommended concentration. The final library yield will indicate the optimal degree of digestion. In particular, this current protocol is optimized for nuclei or extracted DNA, and whole cells will likely require a higher concentration.

Expected yield and curve prior to selection for 50 cells (Note: can skip this visualization step for low yields):
ZymoClean 200ul binding buffer + 50ul reaction. Elute in 15ul TE. Run on HS Bioanalyzer. Yield 4-8ng/ul.

Sequencing suggestion:
Ideally, part A, and B should all be sequenced separately to avoid the fragment length bias of the Illumina sequencer and to recover the most from the single-cell genome.

For cost consideration, part A alone can be sequenced on **NovaSeq X Plus 10B (2x150bp)**, with a 20% PhiX spike-in, which
should be sufficient for determining the single-cell mutation rate.

PROTOCOL MATERIALS

Q5 Reaction Buffer **New England Biolabs Catalog #M0491S** Step 1

META-CS 16 oligos **Integrated DNA Technologies, Inc. (IDT)** Step 1

Transposition Buffer **Diagenode Catalog #C01019043** Step 1

Low TE **Invitrogen Catalog #8019005** Step 1

Triton™ X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #X100-100ML** Step 2

Q5 High GC Enhancer **New England Biolabs Catalog #M0491S** Step 1

16 ADP2 oligos **Integrated DNA Technologies, Inc. (IDT)** Step 1

D5000 ScreenTape **Agilent Technologies Catalog #5067-5588** Step 1

Tagmentase Dilution Buffer **Diagenode Catalog #C01070011** Step 1

Unloaded Tn5 **Diagenode Catalog #C01070010-20** Step 1

Glycerol-500ML **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G5516** Step 1

High Sensitivity D5000 ScreenTape **Agilent Technologies Catalog #5067-5592** Step 1

Zymo DNA Clean & Concentrator Kit **Zymo Research Catalog #D4014** Step 1

16 ADP1 oligos **Integrated DNA Technologies, Inc. (IDT)** Step 1

Q5 polymerase **New England Biolabs Catalog # M0491S** Step 1

1M TRIS pH 8.0 **VWR International Catalog #97062-674** Step 2

Triton™ X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #X100-100ML** Step 2

TL Proteinase K **New England Biolabs Catalog # P8111S** Step 1

Ampure XP beads **Beckman Catalog #A63881** Step 1

Magnesium chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog # 7786-30-3** Step 1

Thermolabile ExoI **New England Biolabs Catalog #M0568S** Step 1

dNTP mix **Thermo Fisher Catalog #R0193** Step 1

UltraPure™ 0.5M EDTA, pH 8.0 **Thermo Scientific Catalog #15575020** Step 2

Water **Invitrogen - Thermo Fisher Catalog #2646318** Step 1

NaCl (5 M), RNase-free **Invitrogen Catalog #AM9760G** In 2 steps

PBS **Invitrogen - Thermo Fisher Catalog #2610807** Step 1

Water **Invitrogen - Thermo Fisher Catalog #2646318** Step 2

Universal Primer/Index Primers **New England Biolabs Catalog #E7335L** Step 1

BSA **Merck MilliporeSigma (Sigma-Aldrich) Catalog # A3294** Step 1

PROTOCOL MATERIALS

1

- Unloaded Tn5 **Diagenode Catalog #C01070010-20** go to step #3
- META-CS 16 oligos **Integrated DNA Technologies, Inc. (IDT)** go to step #2
- Transposition Buffer **Diagenode Catalog #C01019043** go to step #5
- Tagmentase Dilution Buffer **Diagenode Catalog #C01070011** go to step #5
- Q5 Reaction Buffer **New England Biolabs Catalog #M0491S** go to step #7 , #9, #11
- Q5 polymerase **New England Biolabs Catalog # M0491S** go to step #7 , #9, #11
- PBS **Invitrogen - Thermo Fisher Catalog #2610807** go to step #6
- TL Proteinase K **New England Biolabs Catalog # P8111S** go to step #2 , #5
- Q5 High GC Enhancer **New England Biolabs Catalog #M0491S** go to step #7 , #9, #11
- Magnesium chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog # 7786-30-3**
- [M] 100 millimolar (mM) go to step #7
- dNTP mix **Thermo Fisher Catalog #R0193** [M] 10 millimolar (mM) go to step #7 , #9, #11
- BSA **Merck MilliporeSigma (Sigma-Aldrich) Catalog # A3294** [M] 20 mg/mL
- 16 ADP1 oligos **Integrated DNA Technologies, Inc. (IDT)** go to step #7
- 16 ADP2 oligos **Integrated DNA Technologies, Inc. (IDT)** go to step #9
- Water **Invitrogen - Thermo Fisher Catalog #2646318** go to step #7 , #9, #11
- Universal Primer/Index Primers **New England Biolabs Catalog #E7335L** go to step #11
- Thermolabile ExoI **New England Biolabs Catalog #M0568S** go to step #8 , #10
- Low TE **Invitrogen Catalog #8019005**
- Glycerol-500ML **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G5516** go to step #3
- Zymo DNA Clean & Concentrator Kit **Zymo Research Catalog #D4014** go to step #12
- Ampure XP beads **Beckman Catalog #A63881** go to step #12
- High Sensitivity D5000 ScreenTape **Agilent Technologies Catalog #5067-5592**
- D5000 ScreenTape **Agilent Technologies Catalog #5067-5588**

RECIPE FOR MAKING IN-HOUSE REAGENTS

2 **2X Single Cell Lysis Buffer**

- NaCl (5 M), RNase-free **Invitrogen Catalog #AM9760G** [M] 40 millimolar (mM)
- 1M TRIS pH 8.0 **VWR International Catalog #97062-674** [M] 40 millimolar (mM)

Triton™ X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #X100-100ML** [M] 0.3 % volume

Water **Invitrogen - Thermo Fisher Catalog #2646318**

Note

Make 1X Single Cell Lysis Buffer on the day of sort *(30 reactions)*
Add [M] 1.5 µL *TL Proteinase K +* [M] 1.5 µL *1M DTT to* [M] 27 µL *of 2X cell lysis buffer.*
Dilute 1:1 with water to obtain 1X solution for sorting directly into the buffer.

12X quenching solution

NaCl (5 M), RNase-free **Invitrogen Catalog #AM9760G** [M] 600 millimolar (mM)

UltraPure™ 0.5M EDTA, pH 8.0 **Thermo Scientific Catalog #15575020** [M] 90 millimolar (mM)

Triton™ X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #X100-100ML** [M] 0.02 % volume

Note

**Before use prepare 6X Stop Mix for use for 20 reactions below:*

- Dilute [M] 1 µL of TL proteinase K + [M] 19 µL PBS
- Add [M] 20 µL 12X stop solution for the final 6X Stop solution.

ADP1 and ADP2 Mix

1. Reconstitute the 16 ADP1 and 16 ADP2 primers separately in low TE and store in aliquots at -80 °C until ready for use.
2. Make an equimolar mix of the 16 ADP1 and ADP2 primers to make the ADP1 and ADP2 mix respectively.

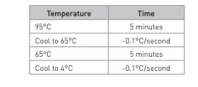
Note

[M] 6.25 micromolar (µM) *each primer x 16 primers for total* [M] 100 micromolar (µM) *solution*

TRANSPOSOME LOADING

3 **Transposon Annealing** 30m

- Reconstitute 16 META-CS oligos and 1 reverse oligo to [M] 100 micromolar (µM) in Annealing Buffer ([M] 40 millimolar (mM) Tris-HCl (pH 8), [M] 50 millimolar (mM) NaCl)
- Combine 1:1 of a singular META-CS oligo with the reverse oligo (there should be 16 separate reactions to put on the thermocycler). Mix up the reaction, spin it down briefly, and run the thermocycler using the conditions below:



Temperature	Time
95°C	5 minutes
Cool to 65°C	-0.1°C/second
65°C	5 minutes
Cool to 4°C	-0.1°C/second

Transposon Assembly.

- Combine all 16 reactions into one tube and aliquot for storage at -80 °C.
- Take [M] 10 µL of this aliquot and combine it with 10µL of unloaded Transposome
- Incubate at 23 °C for 00:30:00
- Add [M] 10 µL of 100% glycerol.
- Aliquot and store at -80 °C.

Note

Estimated final concentration including glycerol storage (~16.7 µM dimerized Tn5)
**Prior to use, dilute Tn5 in Diagenode Tn5 dilution buffer depending on the desired concentration*

Optimization of Tn5 concentration:
Check on 50 cells using dilutions of 1:500, 1:750, 1:1000, and 1:1500, and check the tagmentation curve. go to step #14 Appendix-2

TN5-DUPLEX LIBRARY PROCEDURE

3h 5m 30s

4 **Sorting and lysing cells** [M] 2 µL — 1h 10m

- Prepare nuclei for sorting.
- Sort cells directly into [M] 2 µL of 1X cell lysis buffer

Run the thermocycler using the conditions below 65 °C Lid Temp

30 °C **for** 01:00:00
55 °C **for** 00:10:00

4 °C **hold**

The plate can be stored after lysis.

5 Tn5 tagmentation [M] 8 µL

15m

- Add [M] 8 µL transposition mix (total [M] 10 µL reaction). Vortex, spin down.
- [M] 5 µL Diagenode 2X Tagmentation buffer
- [M] 1 µL diluted Tn5 per optimized dilution instructions above
- [M] 2 µL H2O

Incubate in thermocycler using the conditions below 65 °C Lid Temp

55 °C **for** 00:15:00
4 °C **hold**

6 Quenching [M] 2 µL

40m

- Prepare 6X Stop Mix and add [M] 2 µL Mix per tube. Spin down, vortex, and spin down.
- Incubate in thermocycler using the conditions below 65 °C Lid Temp

37 °C **for** 00:30:00
55 °C **for** 00:10:00
4 °C **hold**

7 First Strand tagging [M] 13 µL

9m 30s

Add [M] 13 µL Strand Tagging Mix 1. Vortex and spin down.

- [M] 5 µL Q5 Reaction Buffer
- [M] 5 µL µL Q5 High GC Enhancer
- [M] 0.6 µL [M] 100 millimolar (mM) MgCl2
- [M] 0.5 µL [M] 10 millimolar (mM) dNTP mix
- [M] 0.25 µL BSA 20mg/ml
- [M] 0.25 µL Q5 polymerase
- [M] 0.85 µL [M] 100 micromolar (µM) ADP1 primer mix
- [M] 0.55 µL H2O

Incubate in thermocycler using the conditions below 105 °C Lid Temp

72 °C for 00:03:00
98 °C for 00:00:30
62 °C for 00:05:00
72 °C for 00:01:00
4 °C hold

8 **Stop reaction** [M] 1 µL 20m

Add [M] 1 µL Thermolabile ExoI per tube. Try to touch the minimum of the solution surface. Spin down first, then plate mix, and spin down again.

37 °C for 00:15:00
65 °C for 00:05:00
4 °C hold 75 °C Lid Temp

9 **Second Strand tagging** [M] 4 µL 6m 30s

Add [M] 4 µL Strand Tagging 2 Mix (total [M] 30 µL). Vortex and spin down.

- [M] 1 µL Q5 Reaction Buffer
- [M] 1 µL Q5 High GC Enhancer
- [M] 0.95 µL [M] 100 micromolar (µM) ADP2 primer mix
- [M] 0.1 µL [M] 10 millimolar (mM) each dNTP mix
- [M] 0.1 µL Q5 polymerase
- [M] 0.85 µL H2O

Incubate in thermocycler using the conditions below 105 °C Lid Temp.

98 °C for 00:00:30
62 °C for 00:05:00
72 °C for 00:01:00
4 °C hold

10 **Stop reaction** [M] 1 µL 20m

Add [M] 1 µL Thermolabile ExoI per tube. Try to touch the minimum of the solution surface. Spin down first, then plate mix, and spin down again.

37 °C for 00:15:00

65 °C for 00:05:00 00:00:00
4 °C hold 75 °C Lid Temp

11 **Library prep** [M] 14 µL 7m 30s

1. Make PCR Mix (per cell):
- [M] 5 µL NEB Universal Primer (NEB E7335S, E7500S, E7710S, E7730S)
- [M] 4 µL Q5 Reaction Buffer
- [M] 4 µL Q5 High GC Enhancer
- [M] 0.4 µL [M] 10 millimolar (mM) each dNTP mix
- [M] 0.4 µL water (H2O)
- [M] 0.2 µL Q5 polymerase ***add last**

2.Add [M] 5 µL NEB Index Primer go to step #14 per tube, avoiding touching the liquid.

3.Add [M] 14 µL PCR Mix per tube, avoiding touching the liquid. Vortex and spin down.

4. Incubate in thermocycler using the conditions below

98 °C for 00:00:20
12 cycles of 98 °C for 00:00:10, 72 °C for 00:02:00
72 °C for 00:05:00
4 °C hold

PURIFICATION AND ZYMO CLEAN

12 **Zymo clean** 5m

1. Utilize the Zymo DNA Clean & Concentrator Kit with associated protocol (abbreviated version below).
- For microbulk samples, use 4:1 DNA binding buffer to sample ([M] 200 µL buffer to [M] 50 µL reaction). For single cell samples, pool desired samples first, then measure the total pooled volume and use 4:1 DNA binding buffer to sample volume. For single cell samples, pool 5 cells per spin column. For 50 cell samples, use 1 spin column per sample.
- Add it to the spin column. The maximum volume that the spin column can hold is [M] 800 µL so pooled samples should have to be run through the same column sequentially until all of the liquid has been run through, discarding flowthrough each time.
- Spin for 00:00:30 at maximum speed on the tabletop centrifuge >10,000xg at RT
- Add [M] 200 µL wash buffer (with ethanol added) and centrifuge column

- Repeat wash again
- Add [M] 42 µL x0.1 TE to elute and wait 00:04:00 at room temperature
- Spin for 00:00:30 at maximum speed on the tabletop centrifuge >10,000xg at RT

2. Run [M] 2 µL on High Sensitivity D5000 TapeStation chip.

13 Size Selection (AMPure) [M] 40 µL DNA library

27m

1. Add [M] 22 µL **(0.55X) resuspended AMPure XP beads to** [M] 40 µL **DNA library**. Vortex and spin down. Label the tube as “A”. Incubate for 00:05:00 at RT.
2. Place tube A on a magnetic stand for 00:05:00. Carefully transfer the supernatant to a new tube. Label the new tube as “B”.
3. Size select tube “A” (0.55x AMPure XP beads):

a. Add [M] 200 µL of 80% freshly prepared ethanol to all tubes while in the magnetic stand, then carefully remove and discard the supernatant.

b. repeat the ethanol wash step one more time.

c. Let air dry on magnetic stand for 00:01:00 at RT.

d. Remove the tubes from the magnetic stand. Elute DNA from beads with [M] 12 µL 0.1X TE (for single cell pools) or [M] 18 µL 0.1X TE (for 50 cell pools). Vortex and gently spin down, incubate for 3 min at RT.

e. Place the tubes on the magnetic stand for 00:03:00. Transfer [M] 18 µL of supernatant to a clean tube.

1. Size select tube "B" (0.8x AMPure XP beads):

a. Add [M] 8 µL **AMPure XP beads to DNA solution in tube B (0.15X)**. Vortex and gently spin down. Incubate for 00:05:00 at RT.

b. Place tube B on the magnetic stand for 00:05:00. Remove supernatant from tube B.

i. **IMPORTANT:** save the supernatant in case AmPure did not work! (the DNA will still be in the supernatant)

c. Add [M] 200 µL of 80% freshly prepared ethanol to all tubes while in the magnetic stand, then carefully remove and discard the supernatant.

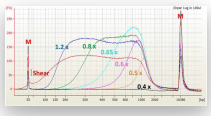
d. repeat the ethanol wash step one more time.

e. Let air dry on magnetic stand for 1 min at RT.

f. Remove the tubes from the magnetic stand. Elute DNA from beads with [M] 12 µL 0.1X TE (for single cell pools) or [M] 18 µL 0.1X TE (for 50 cell pools). Vortex and gently spin down, incubate for 3 min at RT.

g. Place the tubes on the magnetic stand for 00:03:00. Transfer [M] 18 µL of supernatant to a clean tube.

1. Run [M] 2 µL on High Sensitivity D5000 TapeStation chip. (Run [M] 1 µL of sample and 1 of 0.1x TE instead of [M] 2 µL of sample if you need to preserve sample). (Expected concentration for 5 single cell pools is: [M] 300-1000 pg/µL. Expected concentration for 50 cell pools is: [M] 2000-15000 pg/µL .
2. Proceed to DNA quantification with final product and dilute accordingly for sequencing. Run TapeStation, need at least [M] 5 nanomolar (nM) concentration for sequencing. *Fraction B yields best sequencing results.



-
Tube "A" (0.55x) contains DNA fragments with an average of 1000bp.
Tube "B" (0.15x) contains DNA fragments with an average of 400-500bp (fragment size may range from 300bp-600bp).

Appendix-1

14 **SI Appendix Table S1:** Oligonucleotide sequences of META-CS transposon DNA and primers.

All oligos are HPLC purified

	A	B	C
	A	B	
	META transposon sequences:		
	META-CS-1	GGCACCGAAAAAGATGTGTATAAGAGACAG	
	META-CS-2	CTCGGCGATAAAAGATGTGTATAAGAGACAG	
	META-CS-3	GGTGGAGCATAAAGATGTGTATAAGAGACAG	
	META-CS-4	CGAGCGCATTAAAGATGTGTATAAGAGACAG	
	META-CS-5	AGCCCGGTTATAAGATGTGTATAAGAGACAG	
	META-CS-6	TCGGCACCAATAAGATGTGTATAAGAGACAG	

	A	B	C
	META-CS-7	GCCTGTGGATTAAGATGTGTATAAGAGACAG	
	META-CS-8	GCGACCCTTTTAAGATGTGTATAAGAGACAG	
	META-CS-9	GCATGCGGTAATAGATGTGTATAAGAGACAG	
	META-CS-10	GCGTTGCCATATAGATGTGTATAAGAGACAG	
	META-CS-11	GGCCGCATTTATAGATGTGTATAAGAGACAG	
	META-CS-12	ACCGCCTCTATTAGATGTGTATAAGAGACAG	
	META-CS-13	CCGTGCCAAAATAGATGTGTATAAGAGACAG	
	META-CS-14	TCTCCGGGAATTAGATGTGTATAAGAGACAG	
	META-CS-15	CCGCGCTTATTTAGATGTGTATAAGAGACAG	
	META-CS-16	CTGAGCTCGTTTTAGATGTGTATAAGAGACAG	
	META-CS-rev	/5Phos/CTGTCTCTTATACACATC/3InvdT/	
	Adp1 primer mix:		
	META-CS-1-adp1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGCACCGAAAAAGATGTGTATAAG	
	META-CS-2-adp1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTCGGCGATAAAAGATGTGTATAAG	
	META-CS-3-adp1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTGGAGCATAAAGATGTGTATAAG	
	META-CS-4-adp1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGAGCGCATTAAAGATGTGTATAAG	
	META-CS-5-adp1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCCCGGTTATAAGATGTGTATAAG	
	META-CS-6-adp1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGGCACCAATAAGATGTGTATAAG	

	A	B	C
	META-CS-7-adp1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCTGTGGATTAAGATGTGTATAAG	
	META-CS-8-adp1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCGACCCTTTTAAGATGTGTATAAG	
	META-CS-9-adp1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCATGCGGTAATAGATGTGTATAAG	
	META-CS-10-adp1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCGTTGCCATATAGATGTGTATAAG	
	META-CS-11-adp1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGCCGCATTTATAGATGTGTATAAG	
	META-CS-12-adp1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACCGCCTCTATTAGATGTGTATAAG	
	META-CS-13-adp1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCGTGCCAAAATAGATGTGTATAAG	
	META-CS-14-adp1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTCCGGGAATTAGATGTGTATAAG	
	META-CS-15-adp1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCGCGCTTATTTAGATGTGTATAAG	
	META-CS-16-adp1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGAGCTCGTTTTAGATGTGTATAAG	
	Adp2 primer mix:		
	META-CS-1-adp2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGCACCGAAAAAGATGTGTATAAG	
	META-CS-2-adp2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTCGGCGATAAAAGATGTGTATAAG	

	A	B	C
	META-CS-3-adp2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGTGGAGCATAAAGATGTGTATAAG	
	META-CS-4-adp2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGAGCGCATTAAAGATGTGTATAAG	
	META-CS-5-adp2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGCCCGGTTATAAGATGTGTATAAG	
	META-CS-6-adp2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCGGCACCAATAAGATGTGTATAAG	
	META-CS-7-adp2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCCTGTGGATTAAGATGTGTATAAG	
	META-CS-8-adp2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCGACCCTTTTAAGATGTGTATAAG	
	META-CS-9-adp2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCATGCGGTAATAGATGTGTATAAG	
	META-CS-10-adp2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCGTTGCCATATAGATGTGTATAAG	
	META-CS-11-adp2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGCCGCATTTATAGATGTGTATAAG	
	META-CS-12-adp2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTACCGCCTCTATTAGATGTGTATAAG	
	META-CS-13-adp2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCGTGCCAAAATAGATGTGTATAAG	
	META-CS-14-adp2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCTCCGGGAATTAGATGTGTATAAG	
	META-CS-15-adp2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCGCGCTTATTTAGATGTGTATAAG	

	A	B	C
	META-CS-16-adp2	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTGAG CTCGTTTTAGATGTGTATAAG	

Indexes

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