Sequencing of Cell3™ Target Captured Libraries

Libraries enriched by targeted capture using Cell3™Target technology are ready for sequencing on Illumina platforms (such as MiniSeq, MiSeq, NextSeq, HiSeq and NovaSeq instruments).

Calculate captured library molar concentration

An accurate molar concentration can be calculated in the following ways:

- In combination with fluorometric assay reading: use the following formula to calculate molarity.

$$concentration\ in\ nM = \frac{concentration\ in\ ng/ul}{(660\frac{g}{mol}x\ average\ library\ size\ in\ bp)}\ x\ 10^6$$

- In combination with the KAPA Library Quantification – Illumina/Universal kit or equivalent: insert the average fragment size in bp into the required field of the KAPA Library Quantification Data Analysis worksheet (or equivalent from other supplier) to determine library molar concentration.

Choice of Illumina sequencing platform and kit size

Cell3™Target technology is suitable for sequencing on the Illumina MiniSeq, MiSeq, NextSeq, HiSeq and NovaSeq platforms. The recommended cycling parameters for cfDNA libraries are 2x75 paired end sequencing, given that the average cfDNA fragment length is 166 bp. However, longer sequencing reads can be chosen for gDNA and FFPE DNA libraries prepared at larger fragment sizes. Irrespectively of the cycling parameters chosen, the Cell3™Target technology requires paired end sequencing with dual indexing to be performed. The latter is necessary for sample de-multiplexing and use of UMIs and requires 25 sequencing cycles (17 for I7 index/barcode sequencing + 8 for I5 index sequencing). In every Illumina sequencing kit, a certain quantity of reagent excess is provided to allow for sequencing of indexes. However, the amount of excess reagent varies between kit sizes, so it is important to be aware of the maximum amount of sequencing cycles which can be performed for the selected sequencing kit. Table-1 (below) outlines available kit sizes for each compatible Illumina platform; the excess amount of cycles included; and the maximum sequencing read length which can be selected when using Cell3™Target technology in combination with the Illumina UMI Adapters – 16, 48 or 06 reactions format.

Reagent type	Kit size	Excess cycles provided	Max number of cycles	Cell3™Target dual indexing cycles	Max usable sequencing cycles
MiniSeq	75	16	91	25	2x 33
	150	16	166	25	2x 70
	300	16	316	25	2x 145
MiSeq – v2	50	25	75	25	2x 25
	300	25	325	25	2x 150
	500	25	525	25	2x 250
MiSeq – v3	150	25	175	25	2x 75
	600	25	625	25	2x 300
NextSeq 500/550 – v2	75	16	91	25	2x 33
	150	16	166	25	2x 70
	300	16	316	25	2x 145
HiSeq TruSeq SBS v3	50	8	58	25	2x 16
	200	9	209	25	2x 92
HiSeq TruSeq SBS v4	50	25	75	25	2x 25
	250	25	275	25	2x 125
HiSeq Rapid SBS v2	50	24	74	25	2x 24
	200	25	225	25	2x 100
	500	25	525	25	2x 250
HiSeq 3000/4000 SBS	50	24	74	25	2x 24
	150	24	174	25	2x 74
	300	25	325	25	2x 150
NovaSeq 6000 S2	100	25	125	25	2x 50
	200	25	225	25	2x 100
	300	25	325	25	2x 150
NovaSeq 6000 S4	300	25	325	25	2x 150

Table-1: breakdown of kit sizes, excess cycles provided and maximum amount of cycles usable for Illumina MiniSeq, MiSeq, NextSeq, HiSeq, NovaSeq sequencing platforms when using Cell3™Target technology in combination with the Illumina UMI Adapters .

Preparing sample sheet for Illumina sequencing

Captured libraries are compatible with the Illumina TruSeqHT protocol and sample sheet for dual indexed libraries. Depending on the ID of the adapters used, DNA library fragments contain the indexes listed in Table-2.

Some Illumina platforms sequence the I5 index on the opposite strand. Therefore, reverse and complement sequences for the I5 index are provided in the Illumina UMI Adapter table.

Index sequences of Illumina UMI adapters

Well position	Adapter ID	I7 index	I5 index (HiSeq 2000/2500, MiSeq, NovaSeq)	I5 index (HiSeq 3000/4000, NextSeq, MiniSeq)
A1	UMIRC_AN01	CTGATCGTNNNNNNNNN	ATATGCGC	GCGCATAT
B1	UMIRC_AN02	ACTCTCGANNNNNNNNN	TGGTACAG	CTGTACCA
C1	UMIRC_AN03	TGAGCTAGNNNNNNNNN	AACCGTTC	GAACGGTT
D1	UMIRC_AN04	GAGACGATNNNNNNNNN	TAACCGGT	ACCGGTTA
E1	UMIRC_AN05	CTTGTCGANNNNNNNNN	GAACATCG	CGATGTTC
F1	UMIRC_AN06	TTCCAAGGNNNNNNNNN	CCTTGTAG	CTACAAGG
G1	UMIRC_AN07	CGCATGATNNNNNNNNN	TCAGGCTT	AAGCCTGA
H1	UMIRC_AN08	ACGGAACANNNNNNNNN	GTTCTCGT	ACGAGAAC
A2	UMIRC_AN09	CGGCTAATNNNNNNNNN	AGAACGAG	CTCGTTCT
B2	UMIRC_AN10	ATCGATCGNNNNNNNNN	TGCTTCCA	TGGAAGCA
C2	UMIRC_AN11	GCAAGATCNNNNNNNNN	CTTCGACT	AGTCGAAG
D2	UMIRC_AN12	GCTATCCTNNNNNNNNN	CACCTGTT	AACAGGTG
E2	UMIRC_AN13	TACGCTACNNNNNNNNN	ATCACACG	CGTGTGAT
F2	UMIRC_AN14	TGGACTCTNNNNNNNNN	CCGTAAGA	TCTTACGG
G2	UMIRC_AN15	AGAGTAGCNNNNNNNNN	TACGCCTT	AAGGCGTA
H2	UMIRC_AN16	ATCCAGAGNNNNNNNNN	CGACGTTA	TAACGTCG
A3	UMIRC_AN17	GACGATCTNNNNNNNNN	ATGCACGA	TCGTGCAT
B3	UMIRC_AN18	AACTGAGCNNNNNNNNN	CCTGATTG	CAATCAGG
C3	UMIRC_AN19	CTTAGGACNNNNNNNNN	GTAGGAGT	ACTCCTAC
D3	UMIRC_AN20	GTGCCATANNNNNNNNN	ACTAGGAG	CTCCTAGT
E3	UMIRC_AN21	GAATCCGANNNNNNNNN	CACTAGCT	AGCTAGTG
F3	UMIRC_AN22	TCGCTGTTNNNNNNNNN	ACGACTTG	CAAGTCGT
G3	UMIRC_AN23	TTCGTTGGNNNNNNNNN	CGTGTGTA	TACACACG
H3	UMIRC_AN24	AAGCACTGNNNNNNNNN	GTTGACCT	AGGTCAAC
A4	UMIRC_AN25	CCTTGATCNNNNNNNNN	ACTCCATC	GATGGAGT
B4	UMIRC_AN26	GTCGAAGANNNNNNNNN	CAATGTGG	CCACATTG
C4	UMIRC_AN27	ACCACGATNNNNNNNNN	TTGCAGAC	GTCTGCAA
D4	UMIRC_AN28	GATTACCGNNNNNNNNN	CAGTCCAA	TTGGACTG
E4	UMIRC_AN29	GCACAACTNNNNNNNNN	ACGTTCAG	CTGAACGT
F4	UMIRC_AN30	GCGTCATTNNNNNNNNN	AACGTCTG	CAGACGTT
G4	UMIRC_AN31	ATCCGGTANNNNNNNNN	TATCGGTC	GACCGATA
H4	UMIRC_AN32	CGTTGCAANNNNNNNNN	CGCTCTAT	ATAGAGCG
A5	UMIRC_AN33	GTGAAGTGNNNNNNNNN	GATTGCTC	GAGCAATC
B5	UMIRC_AN34	CATGGCTANNNNNNNNN	GATGTGTG	CACACATC
C5	UMIRC_AN35	ATGCCTGTNNNNNNNNN	CGCAATCT	AGATTGCG
D5	UMIRC_AN36	CAACACCTNNNNNNNNN	TGGTAGCT	AGCTACCA
E5	UMIRC_AN37	TGTGACTGNNNNNNNNN	GATAGGCT	AGCCTATC
F5	UMIRC_AN38	GTCATCGANNNNNNNNN	AGTGGATC	GATCCACT
G5	UMIRC_AN39	AGCACTTCNNNNNNNNN	TTGGACGT	ACGTCCAA
H5	UMIRC_AN40	GAAGGAAGNNNNNNNNN	ATGACGTC	GACGTCAT
A6	UMIRC_AN41	GTTGTTCGNNNNNNNNN	GAAGTTGG	CCAACTTC

Well position	Adapter ID	I7 index	I5 index (HiSeq 2000/2500, MiSeq, NovaSeq)	I5 index (HiSeq 3000/4000, NextSeq, MiniSeq)
B6	UMIRC_AN42	CGGTTGTTNNNNNNNNN	CATACCAC	GTGGTATG
C6	UMIRC_AN43	ACTGAGGTNNNNNNNNN	CTGTTGAC	GTCAACAG
D6	UMIRC_AN44	TGAAGACGNNNNNNNNN	TGGCATGT	ACATGCCA
E6	UMIRC_AN45	GTTACGCANNNNNNNNN	ATCGCCAT	ATGGCGAT
F6	UMIRC_AN46	AGCGTGTTNNNNNNNNN	TTGCGAAG	CTTCGCAA
G6	UMIRC_AN47	GATCGAGTNNNNNNNNN	AGTTCGTC	GACGAACT
H6	UMIRC_AN48	ACAGCTCANNNNNNNNN	GAGCAGTA	TACTGCTC
A7	UMIRC_AN49	ACAGCTCANNNNNNNNN	GAGCAGTA	TACTGCTC
B7	UMIRC_AN50	GAGCAGTANNNNNNNNN	ACAGCTCA	TGAGCTGT
C7	UMIRC_AN51	AGTTCGTCNNNNNNNNN	GATCGAGT	ACTCGATC
D7	UMIRC_AN52	TTGCGAAGNNNNNNNNN	AGCGTGTT	AACACGCT
E7	UMIRC_AN53	ATCGCCATNNNNNNNNN	GTTACGCA	TGCGTAAC
F7	UMIRC_AN54	TGGCATGTNNNNNNNNN	TGAAGACG	CGTCTTCA
G7	UMIRC_AN55	CTGTTGACNNNNNNNNN	ACTGAGGT	ACCTCAGT
H7	UMIRC_AN56	CATACCACNNNNNNNNN	CGGTTGTT	AACAACCG
A8	UMIRC_AN57	GAAGTTGGNNNNNNNNN	GTTGTTCG	CGAACAAC
B8	UMIRC_AN58	ATGACGTCNNNNNNNNN	GAAGGAAG	CTTCCTTC
C8	UMIRC_AN59	TTGGACGTNNNNNNNNN	AGCACTTC	GAAGTGCT
D8	UMIRC_AN60	AGTGGATCNNNNNNNNN	GTCATCGA	TCGATGAC
E8	UMIRC_AN61	GATAGGCTNNNNNNNNN	TGTGACTG	CAGTCACA
F8	UMIRC_AN62	TGGTAGCTNNNNNNNNN	CAACACCT	AGGTGTTG
G8	UMIRC_AN63	CGCAATCTNNNNNNNNN	ATGCCTGT	ACAGGCAT
H8	UMIRC_AN64	GATGTGTGNNNNNNNNN	CATGGCTA	TAGCCATG
A9	UMIRC_AN65	GATTGCTCNNNNNNNNN	GTGAAGTG	CACTTCAC
B9	UMIRC_AN66	CGCTCTATNNNNNNNNN	CGTTGCAA	TTGCAACG
C9	UMIRC_AN67	TATCGGTCNNNNNNNNN	ATCCGGTA	TACCGGAT
D9	UMIRC_AN68	AACGTCTGNNNNNNNNN	GCGTCATT	AATGACGC
E9	UMIRC_AN69	ACGTTCAGNNNNNNNNN	GCACAACT	AGTTGTGC
F9	UMIRC_AN70	CAGTCCAANNNNNNNNN	GATTACCG	CGGTAATC
G9	UMIRC_AN71	TTGCAGACNNNNNNNNN	ACCACGAT	ATCGTGGT
H9	UMIRC_AN72	CAATGTGGNNNNNNNNN	GTCGAAGA	TCTTCGAC
A10	UMIRC_AN73	ACTCCATCNNNNNNNNN	CCTTGATC	GATCAAGG
B10	UMIRC_AN74	GTTGACCTNNNNNNNNN	AAGCACTG	CAGTGCTT
C10	UMIRC_AN75	CGTGTGTANNNNNNNNN	TTCGTTGG	CCAACGAA
D10	UMIRC_AN76	ACGACTTGNNNNNNNNN	TCGCTGTT	AACAGCGA
E10	UMIRC_AN77	CACTAGCTNNNNNNNNN	GAATCCGA	TCGGATTC
F10	UMIRC_AN78	ACTAGGAGNNNNNNNNN	GTGCCATA	TATGGCAC
G10	UMIRC_AN79	GTAGGAGTNNNNNNNNN	CTTAGGAC	GTCCTAAG
H10	UMIRC_AN80	CCTGATTGNNNNNNNNN	AACTGAGC	GCTCAGTT
A11	UMIRC_AN81	ATGCACGANNNNNNNNN	GACGATCT	AGATCGTC
B11	UMIRC_AN82	CGACGTTANNNNNNNNN	ATCCAGAG	CTCTGGAT
C11	UMIRC_AN83	TACGCCTTNNNNNNNNN	AGAGTAGC	GCTACTCT
D11	UMIRC_AN84	CCGTAAGANNNNNNNNN	TGGACTCT	AGAGTCCA
E11	UMIRC_AN85	ATCACACGNNNNNNNNN	TACGCTAC	GTAGCGTA

Well position	Adapter ID	I7 index	I5 index (HiSeq 2000/2500, MiSeq, NovaSeq)	I5 index (HiSeq 3000/4000, NextSeq, MiniSeq)
F11	UMIRC_AN86	AGAACGAGNNNNNNNNN	CGGCTAAT	ATTAGCCG
G11	UMIRC_AN87	GTTCTCGTNNNNNNNNN	ACGGAACA	TGTTCCGT
H11	UMIRC_AN88	TCAGGCTTNNNNNNNNN	CGCATGAT	ATCATGCG
A12	UMIRC_AN89	CCTTGTAGNNNNNNNNN	TTCCAAGG	CCTTGGAA
B12	UMIRC_AN90	GAACATCGNNNNNNNNN	CTTGTCGA	TCGACAAG
C12	UMIRC_AN91	TAACCGGTNNNNNNNNN	GAGACGAT	ATCGTCTC
D12	UMIRC_AN92	AACCGTTCNNNNNNNNN	TGAGCTAG	CTAGCTCA
E12	UMIRC_AN93	TGGTACAGNNNNNNNNN	ACTCTCGA	TCGAGAGT
F12	UMIRC_AN94	ATATGCGCNNNNNNNNN	CTGATCGT	ACGATCAG
G12	UMIRC_AN95	AGAACGAGNNNNNNNNN	CGGCTAAT	ATTAGCCG
H12	UMIRC_AN96	GTTCTCGTNNNNNNNNN	ACGGAACA	TGTTCCGT

Table-2: list of adapters contained in the Cell3™Target: Illumina UMI Adapter – 48 reactions unique dual index kit. I7 index and I5 index sequences are listed for each adapter. The reverse and complement sequence of the I5 index is also shown for the relevant Illumina platforms. Sequences are unique in the I5 and I7 position to detect sample index skipping. The 9 bp "NNNNNNNNN" sequence stands for the unique molecular identifier (UMI), which is sequenced on the same read as the I7 index and allows PCR/sequencing error removal and single molecule counting.

The following sample sheet templates can be downloaded from https://nonacus.com/cell3tm-target/ and used according to the Illumina platform of interest:

- When sequencing libraries on the HiSeq 2000/2500, MiSeq or NovaSeq, the Cell3™Target –Samplesheet Template (a).csv should be used.
- When sequencing libraries on the HiSeq 3000/4000, NextSeq or MiniSeq, the Cell3™Target –Samplesheet Template (b).csv should be used.

Open the sample sheet template and add the sample libraries ID's in column A (under "Sample_ID") and names in column B (under "Sample_Name") in the rows corresponding to the adapter used in the library preparation procedure (as prepared in section 1.C). Delete rows containing adapters that are not required and fill in the "Investigator Name", the "Experiment Name" and the "Date" fields (optional). Input the amount of sequencing cycles required for read-1 and read-2 (under "[Reads]"). Save the sample sheet with a new name as a .csv file to use on the Illumina sequencer. For sequencing platforms that can

only be set up using BaseSpace or in standalone mode (such as the NextSeq), select standalone mode and make sure to select the correct amount of cycles for the indexes: 8 cycles for the I5 index and 17 cycles for the I7 index.

Prepare captured library for Illumina sequencing

Following Illumina guidelines for the chosen sequencing platform, denature and dilute the captured library to the recommended concentration and load onto the cartridge. Primers for sequencing are included in Illumina sequencing reagents and no additional custom sequencing primers are needed.