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 22 sequencing cycles in combination with the Illumina UMI Adapter – 16 reactions matched dual index kit (14 for I7 index/barcode sequencing + 8 for I5 index sequencing); and 25 sequencing cycles in combination with the Illumina UMI Adapter – 48 reactions unique dual index kit (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 reactions matched dual index kit or with the Illumina UMI Adapters – 48 reactions unique dual index kit.

Reagent type	Kit size	Excess cycles provided	Max number of cycles	Cell3™Target dual indexing cycles (16 kit / 48 kit)	Max usable sequencing cycles (16 kit / 48 kit)
MiniSeq	75	16	91	22 / 25	2x34 / 2x33
	150	16	166	22 / 25	2x72 / 2x70
	300	16	316	22 / 25	2x147 / 2x145
MiSeq – v2	50	25	75	22 / 25	2x26 / 2x25
	300	25	325	22 / 25	2x151 / 2x150
	500	25	525	22 / 25	2x251 / 2x250
MiSeq – v3	150	25	175	22 / 25	2x76 / 2x75
	600	25	625	22 / 25	2x301 / 2x300
NextSeq 500/550 – v2	75	16	91	22 / 25	2x34 / 2x33
	150	16	166	22 / 25	2x72 / 2x70
	300	16	316	22 / 25	2x147 / 2x145
HiSeq TruSeq SBS v3	50	8	58	22 / 25	2x18 / 2x16
	200	9	209	22 / 25	2x93 / 2x92
HiSeq TruSeq SBS v4	50	25	75	22 / 25	2x26 / 2x25
	250	25	275	22 / 25	2x126 / 2x125
HiSeq Rapid SBS v2	50	24	74	22 / 25	2x26 / 2x24
	200	25	225	22 / 25	2x101 / 2x100
	500	25	525	22 / 25	2x251 / 2x250
HiSeq 3000/4000 SBS	50	24	74	22 / 25	2x26 / 2x24
	150	24	174	22 / 25	2x76 / 2x74
	300	25	325	22 / 25	2x151 / 2x150
NovaSeq 6000 S2	100	25	125	22 / 25	2x51 / 2x50
	200	25	225	22 / 25	2x101 / 2x100
	300	25	325	22 / 25	2x151 / 2x150
NovaSeq 6000 S4	300	25	325	22 / 25	2x151 / 2x150

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 – 16 reactions matched dual index kit or the Illumina UMI Adapters – 48 reactions unique dual index kit.

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 below, Table-2 for the Illumina UMI Adapter -16 reaction matched dual index kit; and Table-3 for the Illumina UMI Adapter - 48 reaction unique dual index kit.

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 tables.

Index sequences of Illumina UMI adapters

Adapter ID	I7 index	I5 index (HiSeq 2000/2500, MiSeq, NovaSeq)	I5 index (HiSeq 3000/4000, NextSeq, MiniSeq)
UMIRC_AD01	GACACAGTNNNNNN	GACACAGT	ACTGTGTC
UMIRC_AD02	GCATAACGNNNNNN	GCATAACG	CGTTATGC
UMIRC_AD03	ACAGAGGTNNNNNN	ACAGAGGT	ACCTCTGT
UMIRC_AD04	CCACTAAGNNNNNN	CCACTAAG	CTTAGTGG
UMIRC_AD05	TGTTCCGTNNNNNN	TGTTCCGT	ACGGAACA
UMIRC_AD06	GATACCTGNNNNNN	GATACCTG	CAGGTATC
UMIRC_AD07	AGCCGTAANNNNNN	AGCCGTAA	TTACGGCT
UMIRC_AD08	CTCCTGAANNNNNN	CTCCTGAA	TTCAGGAG
UMIRC_AD09	ACGAATCCNNNNNN	ACGAATCC	GGATTCGT
UMIRC_AD10	AATGGTCGNNNNNN	AATGGTCG	CGACCATT
UMIRC_AD11	CGCTACATNNNNNN	CGCTACAT	ATGTAGCG
UMIRC_AD12	CCTAAGTCNNNNNN	CCTAAGTC	GACTTAGG
UMIRC_AD13	TTGCTTGGNNNNNN	TTGCTTGG	CCAAGCAA
UMIRC_AD14	CCTGTCAANNNNNN	CCTGTCAA	TTGACAGG
UMIRC_AD15	AGCCTATCNNNNNN	AGCCTATC	GATAGGCT
UMIRC_AD16	TGATCACGNNNNNN	TGATCACG	CGTGATCA

Table-2: list of adapters contained in the Cell3™Target: Illumina UMI Adapter – 16 reactions matched 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 matched in the I5 and I7 position to detect sample index skipping. The 6 bp "NNNNNN" 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.

Well position	Adapter ID	I7 index	I5 index (HiSeq 2000/2500, MiSeq, NovaSeq)	I5 index (HiSeq 3000/4000, NextSeq, MiniSeq)
1A	UMIRC_AN01	CTGATCGTNNNNNNNNN	ATATGCGC	GCGCATAT
1B	UMIRC_AN02	ACTCTCGANNNNNNNNN	TGGTACAG	CTGTACCA
1C	UMIRC_AN03	TGAGCTAGNNNNNNNNN	AACCGTTC	GAACGGTT
1D	UMIRC_AN04	GAGACGATNNNNNNNNN	TAACCGGT	ACCGGTTA
1E	UMIRC_AN05	CTTGTCGANNNNNNNNN	GAACATCG	CGATGTTC
1F	UMIRC_AN06	TTCCAAGGNNNNNNNNN	CCTTGTAG	CTACAAGG
1G	UMIRC_AN07	CGCATGATNNNNNNNNN	TCAGGCTT	AAGCCTGA
1H	UMIRC_AN08	ACGGAACANNNNNNNNN	GTTCTCGT	ACGAGAAC
2A	UMIRC_AN09	CGGCTAATNNNNNNNNN	AGAACGAG	CTCGTTCT
2B	UMIRC_AN10	ATCGATCGNNNNNNNNN	TGCTTCCA	TGGAAGCA
2C	UMIRC_AN11	GCAAGATCNNNNNNNNN	CTTCGACT	AGTCGAAG
2D	UMIRC_AN12	GCTATCCTNNNNNNNNN	CACCTGTT	AACAGGTG
2E	UMIRC_AN13	TACGCTACNNNNNNNNN	ATCACACG	CGTGTGAT
2F	UMIRC_AN14	TGGACTCTNNNNNNNNN	CCGTAAGA	TCTTACGG
2G	UMIRC_AN15	AGAGTAGCNNNNNNNNN	TACGCCTT	AAGGCGTA
2H	UMIRC_AN16	ATCCAGAGNNNNNNNNN	CGACGTTA	TAACGTCG
3A	UMIRC_AN17	GACGATCTNNNNNNNNN	ATGCACGA	TCGTGCAT
3B	UMIRC_AN18	AACTGAGCNNNNNNNNN	CCTGATTG	CAATCAGG
3C	UMIRC_AN19	CTTAGGACNNNNNNNNN	GTAGGAGT	ACTCCTAC
3D	UMIRC_AN20	GTGCCATANNNNNNNNN	ACTAGGAG	CTCCTAGT
3E	UMIRC_AN21	GAATCCGANNNNNNNNN	CACTAGCT	AGCTAGTG
3F	UMIRC_AN22	TCGCTGTTNNNNNNNNN	ACGACTTG	CAAGTCGT
3G	UMIRC_AN23	TTCGTTGGNNNNNNNNN	CGTGTGTA	TACACACG
3H	UMIRC_AN24	AAGCACTGNNNNNNNNN	GTTGACCT	AGGTCAAC
4A	UMIRC_AN25	CCTTGATCNNNNNNNNN	ACTCCATC	GATGGAGT
4B	UMIRC_AN26	GTCGAAGANNNNNNNNN	CAATGTGG	CCACATTG
4C	UMIRC_AN27	ACCACGATNNNNNNNNN	TTGCAGAC	GTCTGCAA
4D	UMIRC_AN28	GATTACCGNNNNNNNNN	CAGTCCAA	TTGGACTG
4E	UMIRC_AN29	GCACAACTNNNNNNNNN	ACGTTCAG	CTGAACGT
4F	UMIRC_AN30	GCGTCATTNNNNNNNNN	AACGTCTG	CAGACGTT
4G	UMIRC_AN31	ATCCGGTANNNNNNNNN	TATCGGTC	GACCGATA
4H	UMIRC_AN32	CGTTGCAANNNNNNNNN	CGCTCTAT	ATAGAGCG
5A	UMIRC_AN33	GTGAAGTGNNNNNNNNN	GATTGCTC	GAGCAATC
5B	UMIRC_AN34	CATGGCTANNNNNNNNN	GATGTGTG	CACACATC
5C	UMIRC_AN35	ATGCCTGTNNNNNNNNN	CGCAATCT	AGATTGCG
5D	UMIRC_AN36	CAACACCTNNNNNNNNN	TGGTAGCT	AGCTACCA
5E	UMIRC_AN37	TGTGACTGNNNNNNNNN	GATAGGCT	AGCCTATC
5F	UMIRC_AN38	GTCATCGANNNNNNNNN	AGTGGATC	GATCCACT
5G	UMIRC_AN39	AGCACTTCNNNNNNNNN	TTGGACGT	ACGTCCAA
5H	UMIRC_AN40	GAAGGAAGNNNNNNNNN	ATGACGTC	GACGTCAT
6A	UMIRC_AN41	GTTGTTCGNNNNNNNNN	GAAGTTGG	CCAACTTC
6B	UMIRC_AN42	CGGTTGTTNNNNNNNNN	CATACCAC	GTGGTATG

Well position	Adapter ID	I7 index	I5 index (HiSeq 2000/2500, MiSeq, NovaSeq)	I5 index (HiSeq 3000/4000, NextSeq, MiniSeq)
6C	UMIRC_AN43	ACTGAGGTNNNNNNNNN	CTGTTGAC	GTCAACAG
6D	UMIRC_AN44	TGAAGACGNNNNNNNNN	TGGCATGT	ACATGCCA
6E	UMIRC_AN45	GTTACGCANNNNNNNNN	ATCGCCAT	ATGGCGAT
6F	UMIRC_AN46	AGCGTGTTNNNNNNNNN	TTGCGAAG	CTTCGCAA
6G	UMIRC_AN47	GATCGAGTNNNNNNNNN	AGTTCGTC	GACGAACT
6H	UMIRC_AN48	ACAGCTCANNNNNNNNN	GAGCAGTA	TACTGCTC

Table-3: 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 samplesheet templates can be provided on request from support@nonacus.com and used according to the Illumina platform of interest:

- When sequencing libraries on the HiSeq 2000/2500, MiSeq or NovaSeq:
  - Cell3™Target – 16 Adapters Samplesheet Template (a).csv should be used in combination with the Illumina UMI Adapter – 16 reaction matched dual index kit;
  - Cell3™Target – 48 Adapters Samplesheet Template (a).csv should be used in combination with the Illumina UMI Adapter – 48 reaction unique dual index kit;
- When sequencing libraries on the HiSeq 3000/4000, NextSeq or MiniSeq:
  - Cell3™Target – 16 Adapters Samplesheet Template (b).csv should be used in combination with the Illumina UMI Adapter – 16 reaction matched dual index kit;
  - Cell3™Target – 48 Adapters Samplesheet Template (b).csv should be used in combination with the Illumina UMI Adapter – 48 reaction unique dual index kit;

Open the samplesheet template and add the sample libraries IDs 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. 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; 14 cycles for the I7 index when using the Illumina UMI Adapter – 16 reaction matched dual index kit; 17 cycles for the I7 index when using the Illumina UMI Adapter – 48 reaction unique dual index kit.

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.