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Innovating Epigenetics Solutions

CATS DNA-seq v1.0 for beta testing

Cat. No. C05010060 (12 rxns)

CATS DNA Library Preparation Kit for Illumina®

USER GUIDE

Version 1.0 03_2018

Please read this manual carefully
before starting your experiment

Contents

Introduction

This manual includes the information required before starting a DNA library preparation experiment with the CATS DNA-seq Kit v1.0 for beta testing 12 reactions.

The Diagenode CATS DNA-seq Kit utilizes the Capture and Amplification by Tailing and Switching technology (CATS).

With the CATS approach DNA ligase is not required for library preparation and is therefore optimal for difficult samples in which DNA maybe degraded or limited in quantity. CATS can be used for inputs down to 50 pg.

Kit method overview

* End repair of 3´phosphorylated DNA prior polynucleotide tailing

Kit materials

Description	Cap color	Quantity (12 rxns)	Storage
End-Repair Master Mix (ERMM)	Yellow	12 µl	-20°C
Tailing Mastermix 1 (TMM1)	Red	24 µl	-20°C
Tailing Mastermix 2 (TMM2)	Red	12 µl	-20°C
RT primer H (RTPH)	Purple	12 µl	-20°C
RT primer M (RTPM)	Purple	12 µl	-20°C
Reverse Transcription Reagent (RTR)	Purple	72 µl	-20°C
Template Switching Reagent (TSR)	Purple	24 µl	-20°C
PCR master mix (PMM)	Green	840 µl	-20°C
10 mM Tris-Cl, pH 8.5	Black	850 µl	-20°C
Nuclease-free water	Blue	950 µl	-20°C
CATS Index 1	Clear	30 µl	-20°C
CATS Index 2	Clear	30 µl	-20°C
CATS Index 3	Clear	30 µl	-20°C
CATS Index 4	Clear	30 µl	-20°C
CATS Index 5	Clear	30 µl	-20°C
CATS Index 6	Clear	30 µl	-20°C
CATS Index 7	Clear	30 µl	-20°C
CATS Index 8	Clear	30 µl	-20°C
CATS Index 9	Clear	30 µl	-20°C
CATS Index 10	Clear	30 µl	-20°C
CATS Index 11	Clear	30 µl	-20°C
CATS Index 12	Clear	30 µl	-20°C

Index sequences

Name	Index primer sequence	Expected index primer sequence read
CATS Index 1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTT CCGATC*T	ATCACG
CATS Index 2	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CGATGT
CATS Index 3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TTAGGC
CATS Index 4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TGACCA
CATS Index 5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ACAGTG
CATS Index 6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GCCAAT
CATS Index 7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CAGATC
CATS Index 8	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ACTTGA
CATS Index 9	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GATCAG
CATS Index 10	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TAGCTT
CATS Index 11	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GGCTAC
CATS Index 12	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CTTGTA

(*) = phosphorothioate bond

Required materials not provided

Materials and Reagents

- Gloves
- Single channel pipettes and corresponding nucleases free filter tips: 10 µl, 20 µl, 200 µl, 1000 µl
- DNA AWAY™ decontamination reagent (VWR, 732-2353)
- Nucleases-free tubes: 0.2, 0.5, 1.5 ml
- Table top centrifuge with strip rotor
- Vortex agitator
- Tube holder for 0.2, 0.5, 1.5 ml tubes
- DiaMag 0.2 ml tube magnetic rack (Diagenode, B04000001)
- DiaMag 1.5 ml tube magnetic rack (Diagenode, B04000003)
- Thermal cycler

For post-PCR libraries purification and clean-up:

- Agencourt® AMPure® XP Beads (Beckman Coulter, A63881)
- Absolute ethanol (VWR, 20821.310)
- 1X TE buffer (Sigma, 93283-100ML)

For DNA libraries size and yield estimation:

- Agilent 2100 BioAnalyzer® and Agilent High Sensitivity DNA Kit (Agilent, 5067-4626)
- Qubit® Fluorometer (Thermo Fisher Scientific); Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32851)

Remarks before starting

Sample requirements

The CATS DNA-seq library preparation method has been designed to specifically work with both double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA) with inputs ranging from 5 ng to 50 pg.

DNA fragmentation

In order to get optimal library preparation it is advised to work with DNA fragments in a range of 100-500 bp. To run a proper DNA fragmentation, Diagenode Bioruptor® products line is advised. Please consult respective protocols available online www.diagenode.com/en/categories/bioruptor-shearing-device. For further information on the subject, please contact customer.support@diagenode.com

The following procedure is efficient for both 3'-OH DNA (e.g. fragmentase –digested DNA, cfDNA, bisulfite-converted DNA) and 3'-phosphorylated DNA (e.g. Bioruptor® sheared DNA).

Library clean-up and QC recommendations

After library preparation, we recommend performing the final purification/clean-up in two rounds of AMPure XP beads size selection in order to efficiently remove the excess of PCR primers and the small library fragments which are not of interest. We recommend using a volume of beads equivalent to the volume of library for the size selections.

A complete description of the procedure is given in the CATS DNA-seq library preparation protocol.

Prior to sequencing, we also recommend quantifying the library(-ies) with the Qubit system using the HS dsDNA kit. Furthermore, we advise assessing the size of the final library in a BioAnalyzer run with the HS DNA kit.

Note: the sum of the P5 and P7 adapters + the artificial poly(A) tail is 143 bp. Therefore, one can estimate the size of the inserts contained in the library by subtracting 143 to the average length of the library fragments.

Multiplexing recommendations with indexes included in the kit

With the listed index primers above, we recommend the following combination if multiplexing of the libraries.

Using this table you can multiplex together:

Multiplexing degree	Pooling recommendations for the libraries in the same sequencing run (either lane or flow cell)			
2 samples	INDEX 12	INDEX 6	/	/
3 samples	INDEX 1	INDEX 10	INDEX 11	/
	INDEX 1	INDEX 3	INDEX 7	/
	INDEX 2	INDEX 4	INDEX 8	/
	2-plex option with any other adapter			/
4 samples	INDEX 2	INDEX 9	INDEX 10	INDEX 11
	INDEX 4	INDEX 5	INDEX 6	INDEX 7
	3-plex option with any other adapter			

For 5 - 11-plex pools use 4-plex options with any other available adapters

Sequencing recommendations

In the following section, a list of recommendations is given for the sequencing of CATS DNA libraries:

- CATS DNA libraries are Illumina® compatible and can be sequenced on all platforms. It is preferable to choose platforms with 4-channel chemistry whenever possible (e.g. MiSeq, HiSeq 2500, HiSeq3000/4000)
- Read1 is starting with the template switch motif which is mainly composed of guanine bases. Therefore, the first three sequencing cycles are mainly calling the base (G) which can be challenging during template generation. As a consequence of this phenomenon, it is advised to treat CATS libraries as low complexity libraries and use a certain % of PhiX spike-in. Please advise your sequencing core facility or sequencing provider of this feature.

- Read2 requires the use of the Diagenode's CATS paired-end sequencing primer (for read2) (Cat. No. C17011050). Indeed, the standard Illumina sequencing primer for read2 will hybridize behind the artificial poly(A) tail of the library and therefore, read2 will mainly result in As. Diagenode's CATS paired-end sequencing primer (for read2) is a custom sequencing primer which will hybridize so that the read2 is directly starting at the beginning of the insert. Illumina's standard sequencing primer is used for read1 (either in single-end or paired-end sequencing).

Platform	Single-end sequencing	Paired-end sequencing	% of PhiX to include in the library pool
MiSeq	Illumina standard sequencing primer for read1	Read1: Illumina sequencing primer Read2: CATS paired-end sequencing primer (for read2) (Cat. No. C17011050) as stand-alone (*)	1-5
HiSeq 2500			1-5
NextSeq 500			20
HiSeq 3000/4000			20

(*) Read2: Please do NOT spike a "custom" CATS paired-end sequencing primer (for read2) (Cat. No. C17011050) into the standard Illumina primer (because the standard primer will lead to sequencing of polyA tracts, reducing run quality). PhiX is required and will be read from read1, but will not be read in read2.

Further information on how to treat custom sequencing primer on Illumina platforms can be found following the links below:

https://support.illumina.com/downloads/miseq-system-custom-primers-guide-15041638.html
https://support.illumina.com/downloads/hiseq-using-custom-primers-reference-guide-15061846.html
https://support.illumina.com/downloads/nextseq-500-custom-primers-guide-15057456.html
More information about Optimizing Cluster Density on Illumina Sequencing Systems
https://support.illumina.com/content/dam/illumina-marketing/documents/products/other/miseq-overclustering-primer-770-2014-038.pdf

Trimming of the reads using Cutadapt software

Due to special mechanisms in creating CATS libraries such as template switching and artificial poly(A) tailing, particular trimming procedures are needed to clean the reads prior to mapping. This step is mandatory and not completing or improperly completing this step may result in a low mapping rate of the reads. The most important trimming step is to remove

the first three bases of the reads and the poly(A) tail. To get as clean results as possible, the Ns and the sequencing adapter contaminations can be removed as well, though they occur in the reads with much lower frequency than the poly(A) tail or the template switch motif. Reads less than 18 nt in length are advised to be discarded to avoid ambiguous mapping.

The following commands are recommended to be used with the Cutadapt software to trim reads prior to mapping.

READ 1

```
cutadapt --trim-n -a GATCGGAAGAGCACACGTCTG -a AGAGCACACGTCTG <input.
file> | cutadapt -u 3 -a A{100} --no-indels -e 0.16666666666666666 - |
cutadapt -O 8 --match-read-wildcards -g GTTCAGAGTTCTACAGTCCGACGATCSSS
-m 18 -o <output.file> -
```

READ 2

```
cutadapt --trim-n --match-read-wildcards -n 2 -g T{100} -a
SSSGATCGTCGG -m 18 –o <output.file> <input.file>
```

For paired-end reads both commands Read 1 and Read2 are needed. For single reads please use only the Read1 command. Note that an installation of Cutadapt is necessary. Please note that we provide both commands [CATS_trimming_r1.sh; CATS_trimming_r2.sh] for trimming and their corresponding User Manual available as a download on the CATS Library Preparation Kits product pages under "Documents" https://www.diagenode.com/en/products/view/2969.

Mapping of the reads

Read1 is aligning to the sense strand of the input DNA whereas the read2 if a paired-end configuration is considered is aligning to the antisense strand.

Technical support

Please contact the Diagenode's technical support in case of any query: customer.support@diagenode.com

Protocol

The following protocol describes the preparation of one library. If multiple libraries are prepared in parallel, a simple scale up is needed as all the reagents are already formulated as master mixes.

Please be aware that the first steps of the protocol are not exactly the same depending on the chemical group ending the 3' end of the dsDNA/ ssDNA fragments.

If unsure of the type of chemical group ending the 3' end of the DNA fragments, please refer to the sample requirement section.

If working with single-stranded DNA, it is not necessary to perform step 1. Step 1 is a denaturation step for double stranded DNA.

All the reactions are performed in a thermal cycler with a heated lid at 105°C.

FOR 3'-OH DNA

1. Denature 8 µl dsDNA solution (5 ng – 50 pg) diluted in 10 mM Tris-HCl, pH 8.5 by heating up at 95°C for 1 minute and then place the tube immediately on ice.

2. Add 2 µl Tailing Master Mix 1 (TMM1) to the 8 µl of denatured ssDNA and mix by pipetting.

3. Incubate the solution for 20 minutes at 37°C, and then 20 minutes at 75°C (can be kept on ice afterwards).

4. Proceed to step (6)

FOR 3'-PHOSPHORYLATED DNA

1. Denature 8 µl dsDNA solution (5 ng – 50 pg) diluted in 10 mM Tris-HCl, pH8.5 by heating up at 95°C for 1 minute and then place the tube immediately on ice.

2. Add 1 µl End-Repair Master Mix (ERMM), to the 8 µl of ssDNA and mix by pipetting.

3. Incubate the solution for 20 minutes at 37°C, and then 10 minutes at 75°C and then cool to room temperature (RT).

4. Add 1 µl Tailing Master Mix 2 (TMM2) to the solution and mix by pipetting.

5. Incubate for 20 minutes at 37°C, and then 20 minutes at 75°C (can be kept on ice afterwards).

Then, proceed to step (6).

Procedure below is the same for all types of DNA

6. Add 1 µl of one of the RT primer (RTPH or RTPM) to the solution according to the table below.

DNA input	RT primer
5 ng – 500 pg	RTPH
500 pg – 50 pg	RTPM

7. Incubate for 2 minutes at 72°C, and then 2 minutes at 42°C.

8. Add 6 µl of Reverse Transcription Reagent (RTR) to the solution and mix by pipetting.

9. Incubate for 15 minutes at 42°C.

10. Add 2 µl of Template Switching Reagent (TSR) and mix by pipetting.

11. Incubate for 120 minutes at 42°C and then denature the RTase for

10 minutes at 70°C.

12. Add 70 µl of PCR Master Mix (PMM) to the solution and 10 µl of 10 µM CATS indexing primer for Illumina. Mix by pipetting.

13. Run the PCR:

Depending on input of your DNA sample, we recommend performing from 10 to 17 amplification cycles (for inputs ranging from 5 ng to 50 pg, respectively).

Temperature	Time	Cycle
94°C	30 sec	Initial denaturation
94°C	15 sec	Amplification 10-17 x
62°C	30 sec	
70°C	30 sec	
70°C	10 min	Final extension

14. OPTIONAL but recommended: load 20 µl of the library into 2% agarose gel to check if the amplification is sufficient. If the product band is weak, add 2-4 PCR cycles.

15. Purify and clean the library up with 1X AMPure® XP beads.

a. Add 100 µl of AMPure® XP beads (brought at room temperature) to the library and mix thoroughly by pipetting.
b. Incubate the mixture for 5 minutes at room temperature.
c. Pellet the beads with a magnet and discard the supernatant.
d. Add 200 µl of freshly prepared 80% ethanol to the beads pellet.
e. Let the beads pellet sit with ethanol for 30 seconds at room temperature.
f. Discard the supernatant while the tubes are on the magnetic stand.
g. Repeat the wash steps (D-F).
h. Spin the tube to bring the remaining ethanol down and place the tube in the magnetic rack again.
i. Discard all remaining supernatant.

j. Air dry the beads pellet for 2 minutes while leaving the tube open.

k. Resuspend the beads pellet in 50 µl of water and wait for 2 minutes at room temperature.

l. Pellet the beads with a magnet and collect the supernatant in a new tube.

m. Repeat the cleanup (step B to J) by adding 50 µl (1X) of AMPure® XP beads to the solution but elute the final library in 20 µl of 1X TE buffer.

16. Quantify the purified library with the Qubit® system (HS dsDNA kit)

17. Perform a quality check of the library with a BioAnalyzer® 2100 (or equivalent) instrument (HS DNA kit).

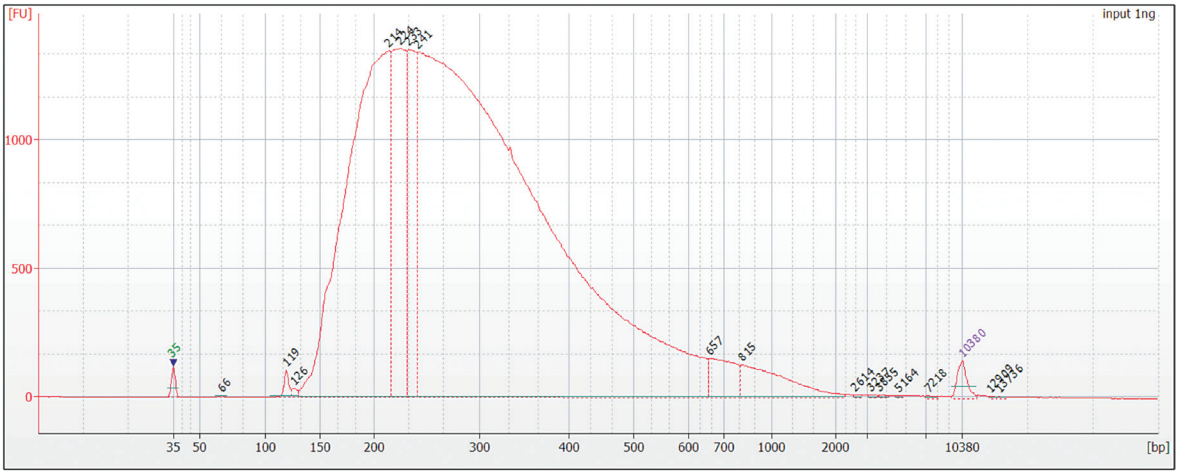


Figure 1. DNA electropherogram of a CATS DNA-seq library made of 1 ng of sheared, reverse cross-linked and purified chromatin following the iDeal protocol for ChIP for Histones (Cat. No. C01010051).

FAQs

Which Illumina sequencing platform should I use when working with CATS DNA-seq libraries?

Diagenode recommends whenever possible to sequence CATS DNA-seq libraries on 4-colour Illumina sequencing platforms (e.g. HiSeq 2500 and MiSeq). However, CATS DNA-seq is also compatible with other Illumina sequencing machines, although particular sequencing recommendations are advised as below.

Sequencing recommendations

Platform	Single-end sequencing	Paired-end sequencing #	% of PhiX to include in the library pool
MiSeq	Illumina standard sequencing primer for read1	Read1: Illumina sequencing primer Read2: CATS paired-end sequencing primer (for read2) (Cat. No. C17011050) as stand-alone (*)	1-5
HiSeq 2500			1-5
NextSeq 500			20
HiSeq 3000/4000			20

 # applies to CATS DNA-seq Kits

(*) Read2: Do NOT spike a "custom" CATS paired-end sequencing primer (for read2) (Cat. No. C17011050) into the standard Illumina primer (because the standard primer will lead to sequencing of polyA tracts, reducing run quality).

Which commands should I use when trimming reads prior to mapping of CATS DNA-seq data?

Due to special construct of CATS libraries including template switching and artificial poly(A) tailing, particular trimming procedures are needed to clean the reads prior to mapping. This step is mandatory -- not completing or improperly completing this step may result in a low mapping rate of the reads.

Here are the commands that should be used with the Cutadapt software to trim reads (SE, PE) prior to mapping.

```
Read1 cutadapt --trim-n -a GATCGGAAGAGCACACGTCTG -a AGAGCACACGTCTG
<input.file> | cutadapt -u 3 -a A{100} --no-indels -e
0.16666666666666666 - | cutadapt -O 8 --match-readwildcards -g
GTTCAGAGTTCTACAGTCCGACGATCSSS -m 18 -o <output.file> -

Read2 cutadapt --trim-n --match-read-wildcards -n 2 -g T{100} -a
SSSGATCGTCGG -m 18 -o <output.file> <input.file>
```

For paired-end reads both commands: Read1 and Read2 are needed. For single reads please use only the Read1 command. Note that an installation of Cutadapt is necessary.

What is the shelf life of CATS DNA-seq kits?

2 years.

If using CATS paired-end sequencing primer for read2, will I also sequence a PhiX control in read2 mode?

No. PhiX is meant to be sequenced with the standard Illumina sequencing primer. CATS paired-end sequencing primer for read2 will not read the PhiX control.

Does the CATS DNA-seq library preparation require working on ice?

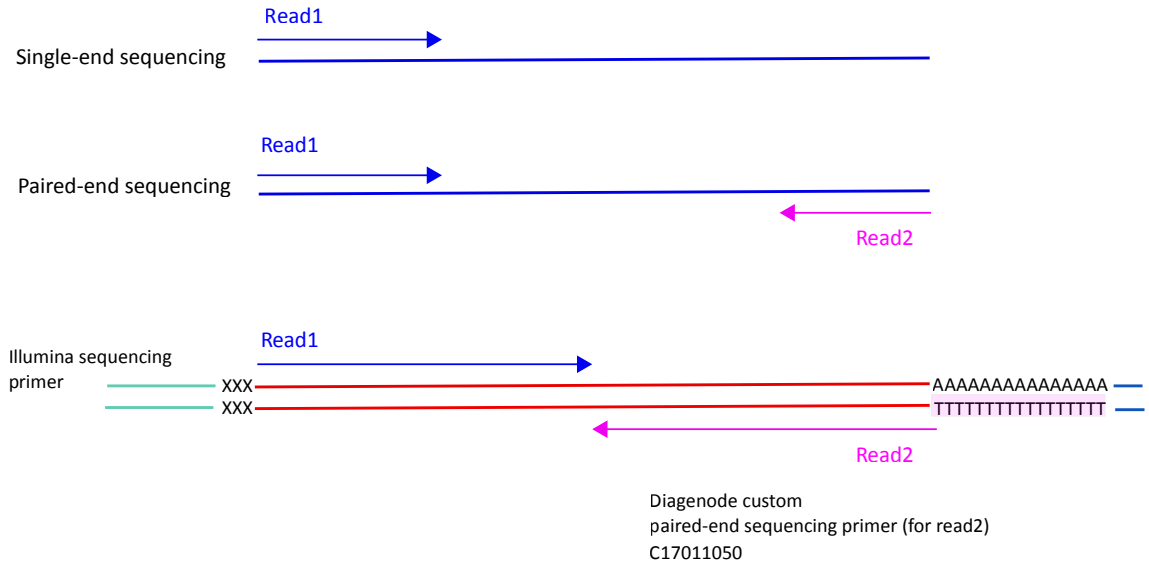
No. The library preparation can be carried on a bench at room temperature. However, reagents and DNA solution which are taken out of the freezer must be placed on ice if not used immediately (once thawed). In the same way, reagents and DNA solution that are no longer being used must be quickly stored back in the freezer. Please consult respective manuals for storage conditions.

Is there any contamination with adapter dimers in CATS DNA-seq libraries?

There is no adapter dimer contamination in CATS DNA-seq libraries, since the technology does not use any ligase. There is no step of ligation, thus adapters cannot be ligated to each other. Additionally there is no "empty library" in the CATS DNA-seq technology.

Does CATS DNA-seq kit work with standard Illumina sequencing primers?

Yes. For single-end sequencing we recommend using the regular Illumina sequencing primer (read 1). For paired-end sequencing the "CATS paired-end sequencing primer (for read2)" is required.



Related products

Product	Cat. No.	Format
CATS Total RNA-seq Kit (with rRNA depletion)	C05010042	24 rxns
CATS mRNA-seq Kit (with polyA selection)	C05010043	24 rxns
CATS RNA-seq Kit	C05010041	24 rxns
CATS Small RNA-seq Kit	C05010040	24 rxns
CATS paired-end sequencing primer	C17011050	50 µl / 500 µl