diagenode

Innovating Epigenetics Solutions

CATS Small RNA-seq kit

CATS Small RNA sequencing kit for Illumina®

Cat. No. **C05010044** (12 rxns)
C05010040 (24 rxns)
C05010048 (96 rxns)

Version 2 I 09.17

Content

MANUAL

Introduction

The CATS Small RNA-seq Kit allows the preparation of DNA libraries for sequencing on Illumina® platforms (MiSeq, HiSeq and NextSeq) from various RNA inputs ranging from 10 pg to 100 ng. The kit allows the sequencing of a wide spectrum of cellular RNAs from 3 nt to 300 nt in size including small non-coding RNAs (miRNA, piRNA, snoRNA and snRNA). The CATS procedure incorporates Illumina®-specific adapters in a highly efficient ligation-independent manner and avoids biases associated with adapter ligation.

The CATS Small RNA-seq Kit utilizes a single-tube method in which sequential addition of several master mixes to the template RNA is possible within 4-5 hours and with a hands-on time of about 30 minutes (depending on the number of samples).

The CATS Small RNA-seq procedure relies on polynucleotide tailing, reverse transcription, a final PCR pre-amplification, and a single library purification step. An optional size selection of the final DNA library can be further performed by SPRI beads or gel purification.

The CATS Small RNA-seq Kit incorporates Illumina® indexes during library amplification and allows for the multiplexing and sequencing of up to 24 libraries on a single flow cell lane.

Kit method overview & time table

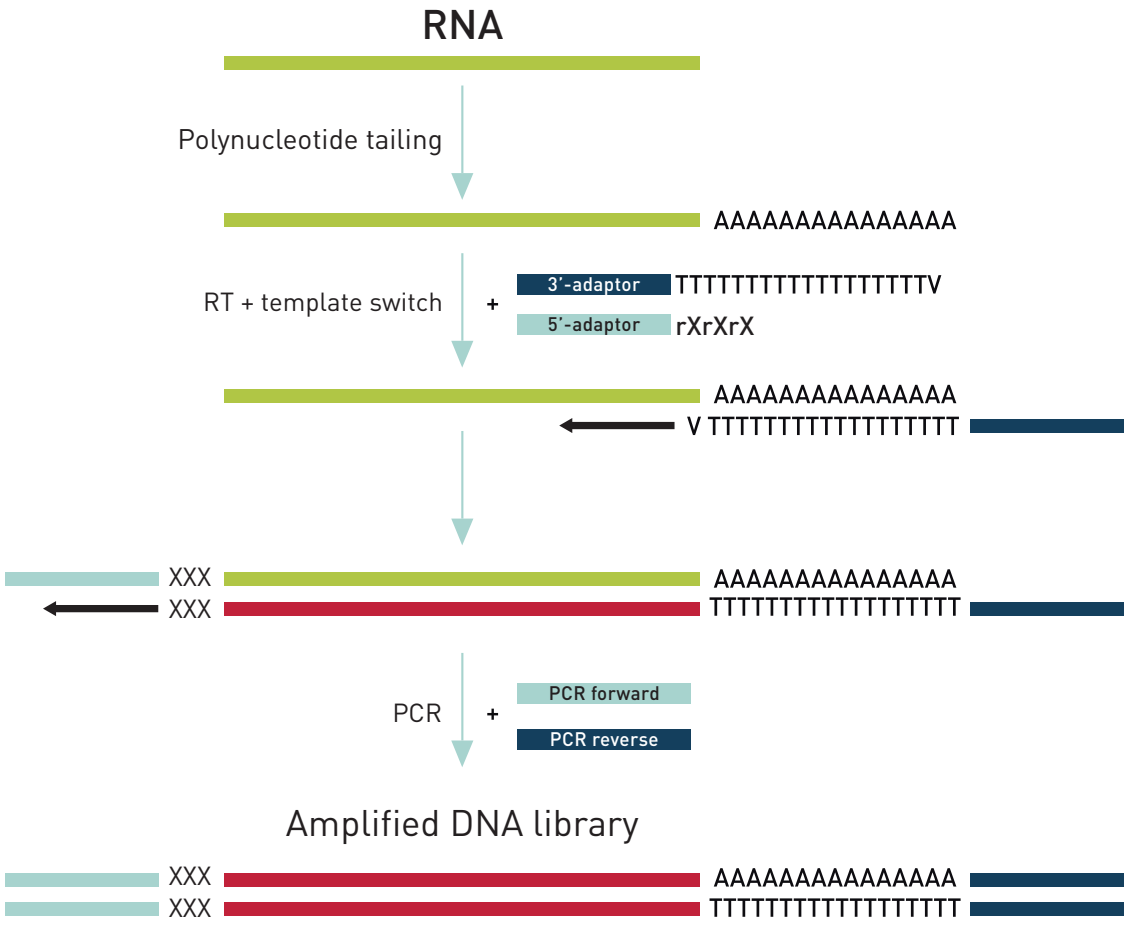

Figure 1: Schematic representation of the workflow used by the CATS Small RNA-seq Kit. Single stranded RNAs are first dephosphorylated (end-repaired) and polyadenylated at the 3'-end. Subsequently, a cDNA strand synthesis is performed in the presence of the anchored poly(dT) oligonucleotide containing terminal P7 Illumina® adaptor sequence. When the reverse transcriptase reaches the 5'-end of the RNA it switches the template and continue DNA synthesis over the template-switching oligonucleotide (TSO). The TSO contains three 3'-terminal ribonucleotides X (rX) which facilitate the template switching and carry the terminal P5 Illumina® adaptor sequence. During PCR pre-amplification of the first cDNA strand, Illumina® adapters carrying P5 and P7 terminal sequences (required for clustering on an Illumina® flow cell) as well as index sequences are incorporated into the library. The sum size of the adapters (the size of "empty" library) is 143 bp.

MANUAL

Kit materials

(*) 96 rxns: if the kit is used for less than 4 times, then aliquoting of the reagents is not necessary. Otherwise, reagents highlighted in red (see below) must be aliquoted in 4 equal parts to be used in future experiments.

Description	Cap color	Quantity (12 rxns)	Quantity (24 rxns)	Quantity (96 rxns)	Storage
Dephosphorylation buffer (DB)	Yellow	24 µL	48 µL	192 µL	-20°C
Dephosphorylation reagent (DR)	Yellow	3.6 µL	7.2 µL	28.8 µL	-20°C
Tailing reagent (TR)	Red	6 µL	12 µL	48 µL	-20°C
Tailing buffer (TB)	Red	12 µL	24 µL	96 µL	-20°C
RT primer H (RTPH)	Purple	12 µL	24 µL	96 µL	-20°C
RT primer M (RTPM)	Purple	12 µL	24 µL	96 µL	-20°C
RT primer L (RTPL)	Purple	12 µL	24 µL	96 µL	-20°C
Reverse Transcription Reagent (RTR)	Purple	72 µL	144 µL	576 µL	-20°C
Template Switching Reagent (TSR)	Purple	24 µL	48 µL	192 µL	-20°C
PCR master mix (PMM)	Green	840 µL	1680 µL	6720 µL	-20°C
CATS Index 1	Blue	30 µL	30 µL	120 µL	-20°C
CATS Index 2	Blue	30 µL	30 µL	120 µL	-20°C
CATS Index 3	Blue	30 µL	30 µL	120 µL	-20°C
CATS Index 4	Blue	30 µL	30 µL	120 µL	-20°C
CATS Index 5	Blue	30 µL	30 µL	120 µL	-20°C
CATS Index 6	Blue	30 µL	30 µL	120 µL	-20°C
CATS Index 7	Blue	30 µL	30 µL	120 µL	-20°C
CATS Index 8	Blue	30 µL	30 µL	120 µL	-20°C
CATS Index 9	Blue	30 µL	30 µL	120 µL	-20°C
CATS Index 10	Blue	30 µL	30 µL	120 µL	-20°C
CATS Index 11	Blue	30 µL	30 µL	120 µL	-20°C
CATS Index 12	Blue	30 µL	30 µL	120 µL	-20°C
CATS Index 13	Blue	/	30 µL	120 µL	-20°C
CATS Index 14	Blue	/	30 µL	120 µL	-20°C
CATS Index 15	Blue	/	30 µL	120 µL	-20°C
CATS Index 16	Blue	/	30 µL	120 µL	-20°C
CATS Index 18	Blue	/	30 µL	120 µL	-20°C
CATS Index 19	Blue	/	30 µL	120 µL	-20°C
CATS Index 20	Blue	/	30 µL	120 µL	-20°C
CATS Index 21	Blue	/	30 µL	120 µL	-20°C
CATS Index 22	Blue	/	30 µL	120 µL	-20°C
CATS Index 23	Blue	/	30 µL	120 µL	-20°C
CATS Index 25	Blue	/	30 µL	120 µL	-20°C
CATS Index 27	Blue	/	30 µL	120 µL	-20°C
Nuclease-free water	Clear	924 µL	800 µL	7392 µL	-20°C
Positive control miRNA	Black	12 µL	12 µL	48 µL	-80°C

Index sequences and multiplexing recommendations

Name	Index primer sequence	Expected index primer sequence read
CATS Index 1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*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
CATS Index 13	CAAGCAGAAGACGGCATACGAGATTGTTGACTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	AGTCAA
CATS Index 14	CAAGCAGAAGACGGCATACGAGATACGGAACTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	AGTTCC
CATS Index 15	CAAGCAGAAGACGGCATACGAGATTCTGACATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ATGTCA
CATS Index 16	CAAGCAGAAGACGGCATACGAGATGGACGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CCGTCC
CATS Index 18	CAAGCAGAAGACGGCATACGAGATGTGCGGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GTCCGC
CATS Index 19	CAAGCAGAAGACGGCATACGAGATCGTTTCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GTGAAA
CATS Index 20	CAAGCAGAAGACGGCATACGAGATAAGGCCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GTGGCC
CATS Index 21	CAAGCAGAAGACGGCATACGAGATTCCGAAACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GTTTCG
CATS Index 22	CAAGCAGAAGACGGCATACGAGATTACGTACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CGTACG
CATS Index 23	CAAGCAGAAGACGGCATACGAGATATCCACTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GAGTGG
CATS Index 25	CAAGCAGAAGACGGCATACGAGATATATCAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ACTGAT
CATS Index 27	CAAGCAGAAGACGGCATACGAGATAAAGGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ATTCCT

(*) = phosphorothioate bond

With the listed index primers above, we recommend the following combinations if the multiplexing of libraries is required:

Multiplexing degree	Combination (C+A+(T) +(S))			
	Index C	Index A	Index T	Index S
2 samples	CATS12	CATS19	/	/
	CATS12	CATS6	/	/
	CATS14	CATS16	/	/
	CATS16	CATS20	/	/
	CATS25	CATS7	/	/
3 samples	CATS1	CATS10	CATS11	/
	CATS2	CATS13	CATS18	/
	CATS4	CATS8	CATS27	/
	CATS5	CATS15	CATS23	/
	CATS7	CATS9	CATS21	/
4 samples	CATS2	CATS9	CATS10	CATS11
	CATS4	CATS5	CATS6	CATS7
	3-plex option with any other adapter			

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

If a higher multiplexing degree is required (>= 12), any combination is possible regardless of the index chosen.

Finally, please note that **each index** is supplied in the kit to be **used up to 3 times** (maximum).

Required materials not provided

General:

- Gloves
- Single channel pipettes and corresponding RNase-free **filter** tips: 10 µL, 20 µL, 200 µL, 1,000 µL
- RNase AWAY™ decontamination reagent (ThermoFisher Scientific, 10328011)
- RNase-free tubes: 0.2, 0.5, 1.5 ml
- Table top centrifuge with strip rotor
- Centrifuge with a speed of 16000 g
- 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:

MicroChIP DiaPure columns (Diagenode, C03040001)

or

Agencourt® AMPure® XP Beads (Beckman Coulter, A63881)

Absolute ethanol (VWR, 20821.310)

1X TE buffer (Sigma, 93283-100ML)

For post-PCR libraries size selection (optional):

Agencourt® AMPure® XP Beads (Beckman Coulter, A63881)

Absolute ethanol (VWR, 20821.310)

1X TE buffer (Sigma, 93283-100ML)

or

4% E-Gel EX Agarose Gels (Thermo Fisher Scientific, G401004) with the required supplementary MinElute® Gel Extraction Kit (Qiagen)

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)

General recommendations

- **Please read the complete manual before first time use**
- Decontaminate the working area as well as all the tools used to perform the library preparation procedure with RNase AWAY™ reagent.
- Wear gloves at all steps to protect the RNA sample from degradation by contaminants and nucleases. Change gloves between different steps throughout the protocol.
- All containers and storage areas must be free of contaminants and nucleases.
- Add enzymes to reaction solutions last and thoroughly incorporate them by pipetting up and down the solution several times.
- When using the protocol for the first time for a definite type of sample, we strongly recommend using the positive miRNA control included in the CATS kit.

Sample requirements

Template

Both total RNA and small RNA can be used as inputs for the CATS reaction.

Small RNAs can be isolated either from biological fluids (e.g. blood plasma or serum), exosomes or from cells using a column-based method (e.g. miRNAeasy Mini Kit (Qiagen)) capable of isolating the small RNA fraction (<200 nt). Be aware that not all the products on the market are able to efficiently isolate RNAs under 200 nt.

For total RNA samples, we strongly recommend to first perform a quality check of the RNA using the Bioanalyzer® 2100 instrument with the associated Agilent RNA 6000 Pico Kit (Agilent, 5067-1531). The CATS kit has been developed with high-quality total RNA (RIN > 8) but the kit can also perform well on partially and even highly degraded samples (e.g. FFPE samples). However, please be aware that sample degradation may result in the underrepresentation of the small RNAs in the final library. By using the default CATS protocol both 3'-OH and 3'-phosphorylated RNAs will be incorporated into the final DNA library. For some applications (e.g miRNA-seq), the presence of 3'-phosphorylated RNAs might be unnecessary or unwanted. To capture only 3'-OH RNAs please modify the protocol as describe in end of the "Detailed protocol" section (*).

Input

Input used for the CATS library preparation needs to be free of contaminants and the RNA must be solubilized in nuclease free water (preferably) or 1X TE buffer.

Positive control: cel-miR-39-3p

Positive control (black cap) is supplied for 12 reactions at a concentration of 1ng/µL. Please, aliquot the control at a final concentration of 125 pg/µL at the first time use and store it at -20°C in order to avoid freeze/thaw cycle which may decrease the library preparation efficiency. When performing library preparation, please use 8 µL of the diluted positive control (= 8µL*125pg/µL = 1ng). A pre-amplification of 10x PCR cycles should be enough to prepare the library.

The miRNA is derived from *Caenorhabditis elegans* and is 22 nt long.

5'-UCACCGGGUGUAAAUCAGCUUG-3'

Sequencing recommendations

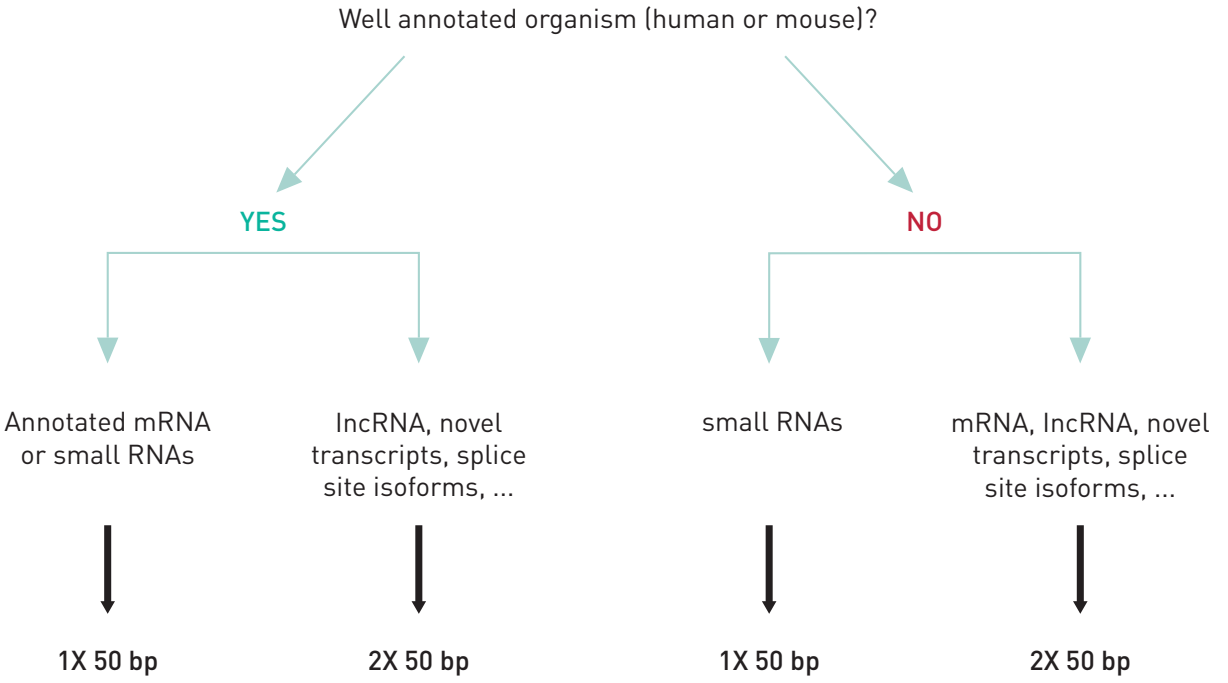

Figure 2: sequencing recommendations for Illumina® platform

The preparation of the CATS library is using the same 5'-end adaptor sequence as the NEBNext small RNA-seq kit. Therefore, it is strongly advised to submit the CATS libraries for sequencing as NEBNext small RNA libraries. Here below is a representation illustrating the 5' end sequence of the 2nd cDNA strand of a CATS library.

AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGACGATCXXXNNNNNNNNNNN

XXX: template switch motif

NNNNNNNNNNN: insert

CATS small RNA-seq sequencing recommendations: important information

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

We also recommend using the HiSeq Control Software version 2.2.38 or higher if sequencing on an Illumina HiSeq instrument.

Platform	Single end sequencing	% of phiX to include in the library pool
MiSeq	Illumina standard sequencing primer for read1	1-5
HiSeq 2500	Illumina standard sequencing primer for read1	1-5
NextSeq 500	Illumina standard sequencing primer for read1	20
HiSeq 3000/4000	Illumina standard sequencing primer for read1	20

In case of read2: Please do NOT spike a "custom" CATS paired-end sequencing primer (for read2) (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 raw reads

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 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.

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-read-wildcards -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 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: 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 RNA-seq Kits product pages under "Documents" https://www.diagenode.com/en/categories/library-preparation.

Mapping of the reads

Read1 is aligning to the sense strand of the input RNA 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

MANUAL

Short protocol for experienced users

Important notice: Before starting, please be aware that the following protocol is designed to include all types of RNAs in the library with no regards to the 5'-end and 3'-end termination. If the sequencing of **native 5'-phosphate and 3'-hydroxyl small RNAs (e.g. miRNA)** is of particular interest, please find a variant of this protocol under the **"section 4. Quality control of the library"** of the detailed protocol.

1. Prepare dephosphorylation master mix (DMM) by mixing 2 µL Dephosphorylation buffer (DB) (yellow cap) and 0.3 µL Dephosphorylation reagent (DR) (yellow cap). For RNA inputs lower than 1 ng (total RNA) or 100 pg (small RNA fraction), the Dephosphorylation reagent (DR) should be diluted 5 times in RNase-free water.
2. Add 2 µL DMM to 8 µL of template RNA. Mix by pipetting up and down 5 times. Unused DMM can be stored at -20°C and thawed up to 3 times.
3. Incubate 15 min at 37°C on thermal cycler.
4. Meanwhile, prepare tailing master mix (TMM) by mixing 1 µL of Tailing buffer (TB) (red cap) and 0.5 µL Tailing reagent (TR) (red cap).
5. Add 1.5 µL TMM to the reaction tube. Mix by pipetting up and down 5 times.
6. Centrifuge tubes shortly (1-2 sec) at max 16.000g – **VERY IMPORTANT**.
7. Incubate 40 min at 37°C + 20 min at 65° on thermal cycler. Then, the sample can be kept on ice afterwards.
8. Add (purple cap)
   1 µL RT primer H (RTPH) for **100 ng – 10 ng** total RNA or **10 ng – 1 ng** small RNA input
   or 1 µL RT primer M (RTPM) for **10 ng – 1 ng** total RNA or **1 ng – 100 pg** small RNA input
   or 1 µL RT primer L (RTPL) for **1 ng – 100 pg** total RNA or **100 pg – 10 pg** small RNA input. NO NEED TO MIX.
9. Incubate 2 min at 72°C + 2 min at 42°C on thermal cycler.
10. Add 6 µL Reverse Transcription Reagent (RTR) (purple cap) and mix by pipetting up and down 5 times.
11. Incubate on thermal cycler at 42°C for 15 min.
12. Add 2 µL Template Switching Reagent (TSR) (purple cap) and mix by pipetting up and down 5 times.
13. Incubate 120 min at 42°C + 10 min at 70°C on thermal cycler. After reverse transcription, the sample can be kept at +4°C overnight or frozen until further use.
    After reverse transcription, the sample can be kept at +4°C overnight or frozen until further use.
14. Add 10 µL of 10 µM CATS Index (24 indexes) for Illumina® (blue cap) and 70 µL PCR master mix (PMM) (green cap). Mix by pipetting up and down 5 times (with the same tip after adding PMM).
15. PCR reaction:
    Initial denaturation: 94°C for 30 sec
    Amplification cycles: **+ from 8** (100 ng total RNA and 10 ng small RNA inputs) **to 20** (100 pg total RNA and 10 pg small RNA inputs) cycles.
    Cycle:
    (denaturation) 94°C for 15 sec (hybridization) 62°C for 30 sec (elongation) 70°C for 30 sec
    Hold at +4°C or freeze at -20°C until further processing
16. Purify 100 µL of PCR reaction with either MicroChIP DiaPure kit or Agencourt® AMPure® XP beads to remove the remaining PCR primers.
    Important notice: to increase to enrichment level of the library in species of interest, a size selection may be of interest. For that, please refer to the corresponding section of the manual.
17. Perform quality check of the library with Qubit® + Bioanalyzer®.
18. Load to HiSeq or MiSeq.

Detailed protocol

Notice before starting:

The protocol has been developed for inputs ranging from 100 ng to 100 pg of total RNA and ranging from 10 ng to 10 pg for the small RNA fraction (< 200 nt). A starting sample volume of 8 µL is used in the library preparation process. If the volume is lower, please fill to 8 µL with nuclease-free water (clear cap).

It is advised, when performing the protocol for the first time with a particular template, to include a reaction for the provided positive control and a reaction without template as a negative control. The negative control should be included in order to check the absence of contaminants in the working environment.

Furthermore, please be aware that the following protocol is designed to include all types of RNAs in the library with no regards to the 5'-end and 3'-end termination. If the sequencing of **native 5'-phosphate and 3'-hydroxyl small RNAs (e.g. miRNA)** is of particular interest, please find a variant of this protocol under the **"section IV. Quality control of the library"** of the detailed protocol.

The following protocol is described for one sample. If using more than one sample, please scale up accordingly.

Starting material:

- **a.** 100 ng – 100 pg total RNA from cells
- **b.** 10 ng – 10 pg enriched small RNA from cells
- **c.** 10 ng – 10 pg total RNA isolated from biological fluids, exosome, etc.

1. RNA de-phosphorylation and tailing

1. Prepare RNA dephosphorylation master mix (DMM) by mixing 2 µL Dephosphorylation buffer (DB) (yellow cap) and 0.3 µL Dephosphorylation reagent (DR) (yellow cap) in a sterile nuclease-free PCR tube. For RNA inputs lower than 1 ng (total RNA) or 100 pg (small RNA fraction), the Dephosphorylation reagent (DR) reagent should be diluted 5 times in RNase-free water.
2. Mix 8 µL input RNA and 2 µL DMM in a sterile nuclease-free PCR tube. Mix by pipetting up and down 5 times. Unused DMM can be stored at -20°C and thawed up to 3 times.
3. Incubate 15 min at 37°C on thermal cycler.
4. Meanwhile, prepare RNA tailing master mix (TMM) by mixing 1 µL Tailing buffer (TB) (red cap) with 0.5 µL Tailing reagent (TR) (red cap) in a sterile nuclease-free PCR tube.
5. Add 1.5 µL TMM to the reaction tube and mix by pipetting up and down 5 times.
6. Centrifuge tubes for a short time (1-2 sec) at max speed (> 10.000g).
7. Incubate 40 min at 37°C + 20 min at 65°C on thermal cycler.

2. Reverse transcription

8. Add (purple cap)
   1 µL RT primer H (RTPH) for **100 ng – 10 ng** total RNA or **10 ng – 1 ng** small RNA input
   or 1 µL RT primer M (RTPM) for **10 ng – 1 ng** total RNA or **1 ng – 100 pg** small RNA input
   or 1 µL RT primer L (RTPL) for **1 ng – 100 pg** total RNA or **100 pg – 10 pg** small RNA input. NO NEED TO MIX.
9. Incubate 2 min at 72°C + 2 min at 42°C on thermal cycler

10. Add 6 µL Reverse Transcription Reagent (RTR) (purple cap) and mix by pipetting up and down 5 times.
11. Incubate on thermal cycler at 42°C for 15 min.
12. Add 2 µL Template Switching Reagent (TSR) (purple cap) and mix by pipetting up and down 5 times.
13. Incubate 120 min at 42°C + 10 min at 70°C on thermal cycler.
    After reverse transcription, the sample can be kept at +4°C overnight or frozen until further use.

3. PCR pre-amplification and purification

14. Add 10 µL of 10 µM CATS Index (24 indexes) for Illumina® (blue cap) and 70 µL PCR master mix (PMM) (green cap). Mix by pipetting up and down 5 times (with the same tip after adding PMM).
15. Place the tube in a thermal cycler with a heated lid (105°C) and run the following program:

94°C for 30 sec
15 sec at 94°C
30 sec at 62°C ⎫ **8 - 20 cycles (consult the table)**
30 sec at 70°C ⎭
Hold at +4°C or freeze at -20°C until further processing

Input RNA	Amount	Cycles
Total RNA	100 ng	8
	10 ng	12
	1 ng	15
	100 pg	20
Small RNA (<200 nt)	10 ng	8
	1 ng	12
	100 pg	15
	10 pg	20

16. Purify 100 µL of PCR reaction with either MicroChIP DiaPure kit or Agencourt® AMPure® XP beads to remove the remaining PCR primers.

Purification of the DNA library using MicroChIP DiaPure columns

Please refer to the corresponding manual and follow protocol instructions. We recommend solubilizing the DNA in 50 µL of elution buffer.

Purification of the DNA library using AMPure® XP beads

1. Make sure the Agencourt®AMPure® XP beads are at room temperature and are thoroughly mixed.
2. Add 1.8 volumes of Agencourt®AMPure® XP beads to the final PCR product and mix by pipetting up and down. (e.g. add 180 µL of Agencourt®AMPure® XP beads per 100 µL of final PCR reaction).
3. Incubate for 5 minutes at room temperature.
4. Pulse spin the tube and place in a magnetic rack for at least 5 minutes until the beads have collected to the wall of the tube and the solution is clear.

5. Carefully remove and discard the supernatant without disturbing the beads.
6. Keep the tube on the magnet and add 400 µL of **freshly** prepared 80% ethanol.
7. Incubate at room temperature for 30 seconds and then carefully remove and discard the supernatant.
8. Repeat steps 6–7.
9. Pulse spin the tube, return to the magnet and remove any residual ethanol with a pipette.
10. Keep the tube in the magnetic rack with the cap open and air dry the beads for 2-3 minutes at room temperature.
11. Resuspend the beads in 50 µL of 1X TE Buffer.
12. Incubate for 2 minutes at room temperature.
13. Pulse spin the tube and place in a magnetic rack for at least 5 minutes and collect the clear supernatant.
14. Transfer the purified library in a sterile nuclease-free tube.
    Important notice: To increase the enrichment level of the library in species of interest, a size selection may be of interest. For that, please refer to the "Optional enrichment and size selection protocols" section of the manual.

4. Quality control of the library

17. Measure the concentration of the DNA in the purified PCR reaction with Qubit® dsDNA HS Assay Kit.
    Typical amount generated after library preparation are between 500-600 ng if beginning with total RNA and 800-1000 ng if beginning with the small RNA fraction (← 200nt).
18. Load 1 µL of the purified PCR reaction on the Bioanalyzer® using a DNA 1000 chip (for DNA concentration > 5 ng/µL) or DNA High Sensitivity Chip (for DNA concentration < 5 ng/µL) according to the manufacturer's instructions.

(*) To sequence only 3'-OH RNAs (e.g. miRNA), please substitute the steps **1-5** of the Detailed or Short Protocol with the following steps:

1. Prepare a modified RNA tailing master mix (mTMM) by mixing 2 µL Dephosphorylation buffer (DB), 1 µL Tailing buffer (TB) and 0.5 µL Tailing reagent (TR) for each RNA sample in a sterile nuclease-free PCR tube.
2. Combine 8 µL input RNA and 3 µL mTMM in a sterile nuclease-free PCR tube and mix by pipetting up and down 5 times.
3. Follow the Detailed or Short protocol from **step 6**.

Optional enrichment and size selection protocols

After the initial QC of the library using Bioanalyzer®, the samples are ready to be loaded onto Illumina® HiSeq, MiSeq or NextSeq flowcells. However, for some applications (e.g. miRNA-seq, piRNA-seq) it is recommended to size select the final DNA library to exclude sequencing of unnecessary or unwanted RNA species.

There are several different methods for performing enrichment and size selection. Enrichment for short or long fragments can be performed using AMPure® XP beads. If the sequencing of only 18-24 nt short regulatory RNAs (such as miRNA and piRNA) is required, it is recommended to use size selection with 4% E-Gel EX Agarose Gels.

Likewise, if sequencing very short RNAs is unnecessary or unwanted, the final DNA libraries can be enriched for longer fragments with either AMPure® XP Beads or 4% E-Gel EX Agarose Gels.

Those optional size selection procedures are especially meant to be used with **total RNA template**. If the small RNA fraction is used as a template in the library preparation, a simple purification procedure may only be applied to remove the excess of PCR primers.

I. Enrichment of DNA libraries for short (< 50 nt insert size) fragments using AMPure® XP Beads

1. Add 65 µL (1.3X) of resuspended Agencourt® AMPure® XP beads to the purified PCR reaction (50 µL) and mix well on a vortex mixer or by pipetting up and down at least 10 times.
2. Incubate for 5 minutes at room temperature.
3. Pulse spin the tube and place in a magnetic rack for at least 5 minutes until the beads have collected to the wall of the tube and the solution is clear.
4. Carefully **transfer the supernatant** to a new tube and **discard the beads**.
5. Add 185 µL (3.7X) of resuspended AMPure® XP beads to the supernatant (115 µL), mix well and incubate for 5 minutes at room temperature.
6. Pulse spin the tube and place in a magnetic rack for at least 5 minutes until the beads have collected to the wall of the tube and the solution is clear.
7. Carefully remove and discard the supernatant. **Do not discard beads!**
8. Keep the tube on the magnet and add 400 µL of **freshly** prepared 80% ethanol.
9. Incubate at room temperature for 30 seconds and then carefully remove and discard the supernatant.
10. Repeat steps 8–9.
11. Pulse spin the tube, return to the magnet and remove any residual ethanol with a pipette.
12. Keep the tube in the magnetic rack with the cap open and air dry the beads for 2-3 minutes at room temperature.
13. Resuspend the beads in 30 µL of 1X TE buffer.
14. Incubate for 2 minutes at room temperature.
15. Pulse spin the tube and place in a magnetic rack for at least 5 minutes and collect the supernatant.

II. Enrichment of DNA libraries for long (> 50 nt insert size) fragments using AMPure® XP Beads (optional)

1. Add 65 µL (1.3X) of resuspended Agencourt® AMPure® XP Beads to the purified PCR reaction (50 µL) and mix well on a vortex mixer or by pipetting up and down at least 10 times.
2. Incubate for 5 minutes at room temperature.
3. Pulse spin the tube and place in a magnetic rack for at least 5 minutes until the beads have collected to the wall of the tube and the solution is clear.
4. Carefully **remove and discard the supernatant** without disturbing the beads.
5. Keep the tube on the magnet and add 400 µL of freshly prepared 80% ethanol.
6. Incubate at room temperature for 30 seconds and then carefully remove and discard the supernatant.
7. Repeat steps 5–6.
8. Pulse spin the tube, return to the magnet and remove any residual ethanol with a pipette.
9. Keep the tube in the magnetic rack with the cap open and air dry the beads for 2-3 minutes at room temperature.
10. Resuspend the beads in 30 µL of 1X TE buffer.
11. Incubate for 2 minutes at room temperature.
12. Pulse spin the tube and place in a magnetic rack for at least 5 minutes and collect the supernatant.

III. Precise Size Selection of DNA libraries with 4% E-Gel EX Agarose Gels

1. Perform purification of DNA libraries using MicroChIP DiaPure columns but elute in 20 µL of elution buffer.
2. This purification step also works well with the QIAQuick PCR purification kit (Qiagen).
3. Load **20 µL** of eluate into a well of 4% E-Gel EX Agarose Gel and run the gel for 15 min.
4. Remove the gel from the plastic wrap and view on a UV transIlluminator.
5. For miRNAs and piRNAs sequencing, cut the bands corresponding to ~160-180 bp. For longer RNAs isolate the bands corresponding to 180-500 bp.
6. Place the gel slices in a 1.5 ml tube and isolate DNA with the addition of 600 µL of Buffer QG (from MinElute Gel Extraction Kit). **DO NOT FOLLOW THE MANUAL FROM MinElute Gel Extraction Kit**.
7. Incubate the gel slices at 37°C for 10 min and vortex every 3 min until the gel has completely dissolved.
8. Apply sample to the MinElute column (from MinElute Gel Extraction Kit) and centrifuge for 20 sec at 16.000 g.
9. Add 0.75 ml Buffer PE to the MinElute column, incubate for 1 min and centrifuge for 30–60 s.
10. Discard flow-through and place the MinElute column back in the same tube. Centrifuge the column for an additional 1 min.
11. Place MinElute column in a clean 1.5 ml microcentrifuge tube.
12. Add 20 µL Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the MinElute membrane and let the column stand for 2 min.
13. Centrifuge the column for 1 min at 10.000 g and collect the eluate.

MANUAL

Quality requirements

When the CATS protocol is followed precisely and the proper conditions for the particular RNA input is used, the following standards of quality can be expected:

1. The yield of the final DNA libraries in 50 µL eluate after DiaPure purification or AMPure® XP beads purification should be typically between 5 – 20 ng/µL when quantified with a Qubit® HS dsDNA assay.
2. The amount of primer (40-80 bp) or/and "empty" DNA libraries (~143 bp) should not exceed 5-10% of the total DNA libraries.

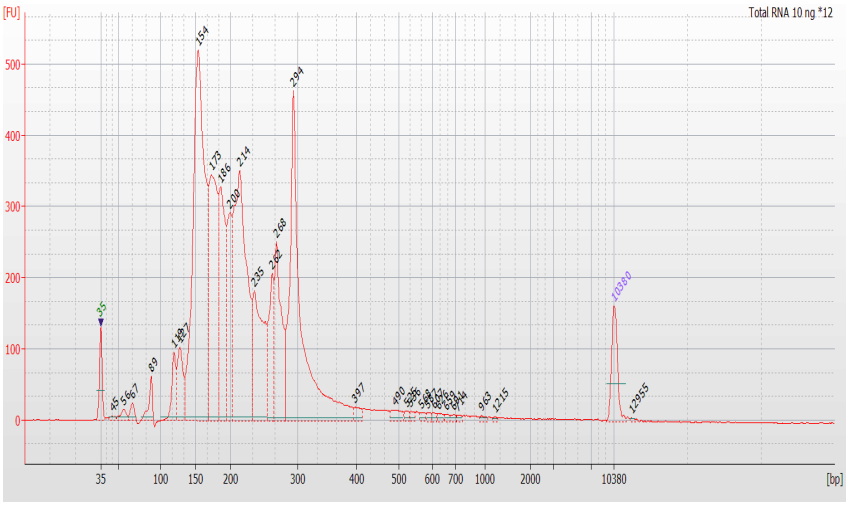

Figure 3: Bioanalyzer® DNA electrophoregram of CATS library made from 10 ng total human universal RNA (Agilent, 740000)

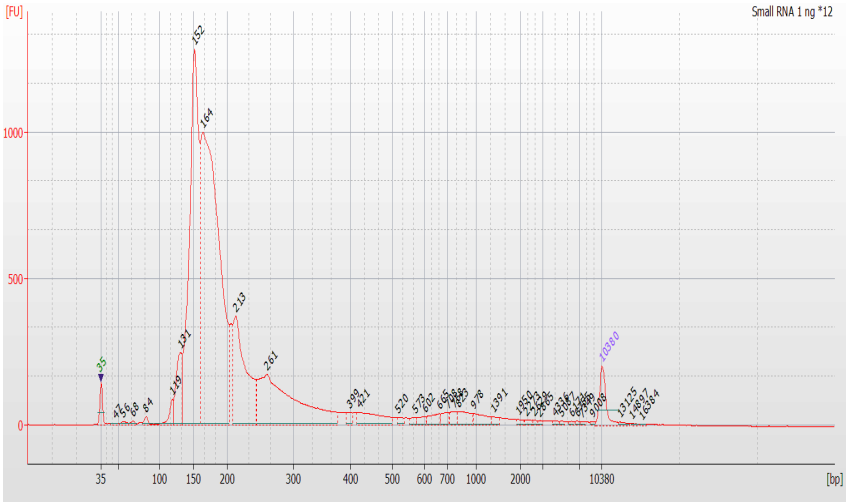

Figure 4: Bioanalyzer® DNA electrophoregram of CATS library made from 1 ng of the small RNA fraction isolated from total human universal RNA (Agilent, 740000)

References

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Turchinovich A , Surowy H, Serva A, Zapatka M, Lichter P, Burwinkel B. Capture and Amplification by Tailing and Switching (CATS): An ultrasensitive ligation-independent method for generation of DNA libraries for deep sequencing from picogram amounts of DNA and RNA // RNA Biol. 2014 Jun 12;11(7).

Related products

Product name	Cat. No.	Format
CATS Total RNA-seq Kit (with rRNA depletion) v2	C05010046 C05010042 C05010050	12 rxns 24 rxns 96 rxns
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Innovating Epigenetics Solutions

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