diagenode

Innovating Epigenetics Solutions

CATS mRNA-seq kit v2 (with polyA selection)

CATS mRNA sequencing kit for Illumina®

Cat. No. **C05010047** (12 rxns)
C05010043 (24 rxns)
C05010051 (96 rxns)

Version 2 I 09.17

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A. POLY(A) RNA ISOLATION

Introduction

Diagenode's poly (A) + RNA module is designed for the isolation of poly (A) + RNA transcripts from total RNA for RNA library preparation and sequencing. The module is is designed for using 1 µm paramagnetic beads coupled with oligo d (T) 25 chains.

This structure is then used to capture RNA bearing a poly (A) queue. This method allows manual processing of several samples at a time and can be also adapted for an automated platform. Additionally, magnetic isolation is a gentle pull-down procedure that allows for intact, full-length mRNA in the eluate at the end of the protocol. Thus the isolated mRNA which is completely representative of the sample population can be obtained in less than 1 hour and can be eluted in a small volume for a highly concentrated template.

Kit materials

Description	Quantity (12 rxns)	Quantity	Quantity (96 rxns)	Storage
Oligo d(T)25 beads	240 µL	480 µL	1920 µL	+4°C
2X RNA binding buffer	3.6 ml	7.2 ml	28.8 ml	+4°C
Wash buffer	7.2 ml	14.4 ml	57.6 ml	+4°C
Tris buffer	804 µL	1.65 ml	6.5 ml	+4°C
Nuclease-free water	600 µL	1.2 ml	4.8 ml	+4°C

Required materials not provided

- Gloves
- Single channel pipettes and corresponding filter tips: 10µl, 20µl, 200µl, 1000µl
- Crushed ice
- RNase AWAY™ decontamination reagent (ThermoFisher Scientific, 10328011)
- Table top centrifuge with strip rotor
- Tube holder for 0.2 ml tubes
- 0.2 ml RNase-free tubes
- DiaMag 0.2 magnetic rack (Diagenode, B04000001)
- Thermal cycler

Protocol

Starting material: 50ng – 5µg of total RNA

1. Dilute the total RNA with nuclease-free water to a final volume of 50 µl in nuclease-free 0.2ml PCR tube.
2. Aliquot 20µl of oligo d(T)25 beads into a nuclease-free 0.2ml tube.
3. Wash the beads two times with 100µl of 2X RNA binding buffer and remove the supernatant.
4. Resuspend the beads in 50µl of 2X RNA binding buffer and add the 50µl of total RNA sample from step 1.
5. Place the tubes on the thermal cycler and heat the sample at 65°C for 5 minutes and hold at 4°C to denature the RNA and facilitate binding of the poly (A)+ RNA to the beads.
6. Remove tubes from the thermal cycler when the temperature reaches +4°C.
7. Resuspend the beads. Pipette up and down slowly 6 times to mix thoroughly.
8. Place the tubes on the bench and incubate at room temperature for 5 minutes to allow the RNA to bind to the beads.
9. Resuspend the beads. Pipette up and down slowly 6 times to mix thoroughly.
10. Incubate for 5 more minutes at room temperature to allow the RNA to bind to the beads.
11. Place the tubes on the magnetic rack at room temperature for 2 minutes to separate the poly (A)+ RNA bound to the beads from the solution.
12. Remove and discard all of the supernatant. Take care not to disturb the beads.
13. Remove the tubes from the magnetic rack.
14. Wash the beads twice with 200 µl of wash buffer to remove the unbound RNA. Pipette the entire volume up and down 6 timers to mix thoroughly.
15. Place the tubes on the magnetic rack at room temperature for 2 minutes.
16. Remove and discard all the supernatant from each tube. Take care not to disturb the beads.
17. Remove tubes from the magnetic rack.
18. Add 50µl of Tris buffer to each tube. Pipette the entire volume up and down 6 timers to mix thoroughly.
19. Place the tubes on the thermal cycler. Close the lid and heat the sample at 80°C for 2 minutes, then hold at 25°C to elute the poly (A)+ RNA from the beads.
20. Remove the tubes from the thermal cycler when the temperature reaches 25°C.
21. Add 50µl of 2X RNA binding buffer to each sample to allow the RNA to bind to the beads. Gently pipette the entire volume up and down 6 times to mix thoroughly.
22. Incubate the tubes at room temperature for 5 minutes.
23. Resuspend the beads. Pipette up and down slowly 6 times to mix thoroughly.
24. Incubate at room temperature for 5 more minutes to allow the RNA to bind to the beads.
25. Place the tubes on the magnetic stand at room temperature for 2 minutes.
26. Remove and discard all of the supernatant from each tube. Take care not to disturb the beads.
27. Remove the tubes from the magnetic stand.
28. Wash the beads once with 200µl of wash buffer. Gently pipette the entire volume up and down 6 times to mix thoroughly.
29. Place the tubes on the magnetic stand at room temperature for 2 minutes.

30. Remove and discard all of the supernatant from each tube. Take care not to disturb the beads.
31. Remove the tubes from the magnetic stand.
32. Elute the mRNA from the beads by adding 7µl of the Tris buffer and incubating the sample at 80°C for 2 minutes. Immediately place the tubes on the magnetic rack.
33. Collect the purified mRNA by transferring the supernatant to a clean nuclease-free PCR tube.
34. Place the tube on ice.
35. Assess the yield and size distribution of the purified mRNA. Run 1µl on the BioAnalyzer® using a RNA 6000 Pico chip.
    This is only possible to some extent. Only if the starting amount is high enough to recover a sufficiently concentrated mRNA solution which can be detected by the BioAnalyzer® system.
    Typically, 1-2% of the starting sample amount is recovered after poly (A)+ selection using this module.
36. Store the isolated mRNA at -80°C or process it directly into the CATS library preparation

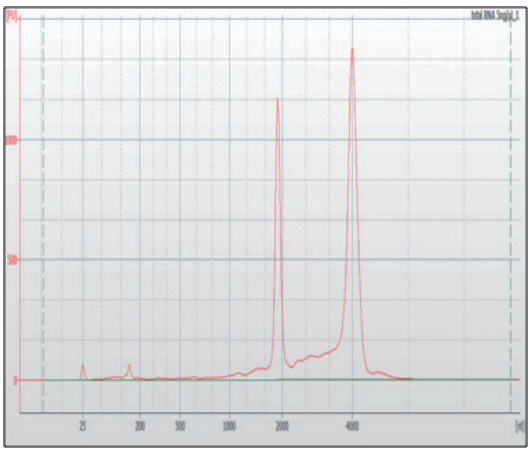

Figure 1: BioAnalyzer® RNA electrophoregram of total RNA. Starting material of the poly (A)+ isolation.

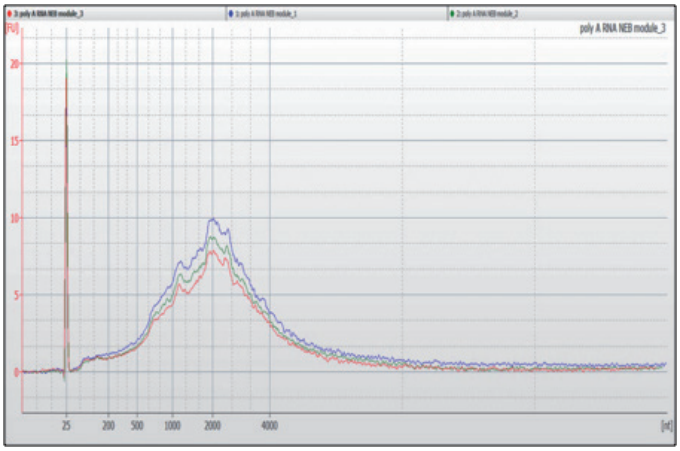

Figure 2: BioAnalyzer® RNA electrophoregram of mRNA. Three technical replicates.

B. CATS LIBRARY PREPARATION

Introduction

CATS RNA-seq kit allows preparation of DNA libraries for sequencing on Illumina® platforms (MiSeq, HiSeq and NextSeq) from various RNA inputs ranging from 100 pg to 10 ng. The kit allows sequencing a wide spectrum of cellular RNAs from total RNA or either isolated with a poly (A) selection or rRNA depletion module. The CATS procedure incorporates Illumina®-specific adapters in a highly efficient ligation-independent manner and avoids biases associated with adapters' ligation.

CATS RNA-seq kit is a single-tube method that is executed by sequential addition of several master mixes to the template RNA sample which can be performed within 4-5 hours and has a hands-on time of about 30 min (depending on the number of samples).

CATS RNA-seq procedure relies on template fragmentation and end repair, polynucleotide tailing, reverse transcription, final PCR pre-amplification and a library purification step. The directionality of the template-switch reaction occurring during the reverse transcription maintains the strand orientation of the template RNA. This feature generates high-quality and strand-specific sequencing data from the synthesized cDNA.

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

MANUAL

Kit method overview & time table

Figure 3: Schematic representation of the workflow used by the CATS RNA-seq Kit. Single-stranded RNAs are first chemically fragmented, 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 reverse transcriptase reaches the 5Ð-end of the RNA, it switches the template and continues DNA synthesis over the template switching oligonucleotide (TSO). The TSO contains three 3Ð-terminal ribonucleotides X (rX) which facilitate the template switching and carry 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.

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
RNA fragmentation buffer	White	12 µL	24 µL	96 µL	-20°C
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 PLUS (TRP)	Red	6 µL	12µL	48 µL	-20°C
Tailing Buffer PLUS (TBP)	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
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 (total human brain RNA)	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:

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)

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

The CATS library preparation method has been developed with high quality, intact RNA template that is free of contaminants. It has been designed to work with either purified total RNA, isolated poly(A) mRNA or rRNA-depleted RNA.

After RNA isolation, if the starting amount is not limited, it is recommended to first perform an RNA integrity check using the Agilent RNA 6000 RNA Pico kit (Cat. 5067-1513).

The CATS library preparation kit also works well with degraded material (e.g. FFPE tissue) but adaptation of the following method has to be made by the final user. Typically, the fragmentation step must be faster, if not completely omitted. We recommend performing a fragmentation time course to find the optimal condition to construct a high-quality library.

The CATS RNA-seq Kit has three different options containing one or none sample preparation module. If mRNA-sequencing is of interest, we recommend the CATS mRNA-seq Kit (Cat. C05010043) with an included poly(A) mRNA isolation module. If whole transcriptome sequencing is of interest, we recommend the CATS total RNA-seq Kit (Cat. C05010042) with an included rRNA depletion module. If total RNA sequencing is of interest or rRNA depletion is not required, we recommend the CATS library preparation core without any sample preparation module (Cat. C05010041).

The CATS library preparation method has been specifically developed to work with the different sample preparation modules (poly(A) mRNA isolation or rRNA depletion). We recommend not making any substitutions for the combined solutions.

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 eventually in 1X TE buffer.

The CATS library preparation kit has been developed with inputs as low as 100 pg (in 7 µl of water). However, if the starting amount is not limited, we recommend using 1-10 ng RNA as the library complexity correlates with the template quantity.

Positive control: total human brain RNA

Positive control (black cap) is supplied for 12 reactions at a ready-to-use concentration of 1 ng/µl. Please carefully store this product at -80°C and aliquot it in order to avoid freeze/thaw cycle which may decrease the library preparation efficiency.

When performing library preparation, please use 1 µl of the positive control (= 1 ng) and complete the solution with 6 µl of nuclease-free water before performing the first step of the protocol (fragmentation). A pre-amplification of 10x PCR cycles should be enough to prepare the library.

Sequencing recommendations

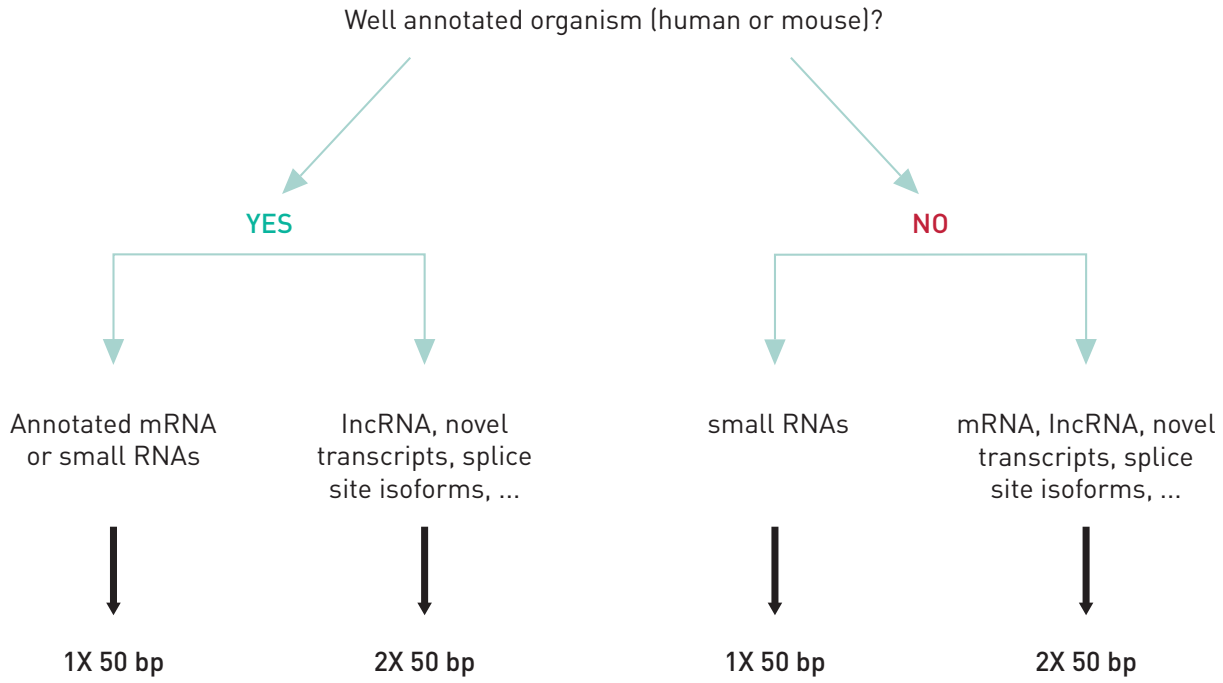

Figure 4: sequencing recommendations for Illumina® platform

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

If considering paired-end sequencing, it is advised to use CATS paired-end sequencing primer (for read2) C17011050 as it is specifically designed to span the poly(A) tail of the library and thus directly sequence the insert.

Then, if using standard Illumina sequencing primer for read2, a significant portion of the read2 will in fact results only in As.

CATS v2 mRNA-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	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) (C17011050) as stand-alone (*)	1-5
HiSeq 2500	Illumina standard sequencing primer for read1	Read1: Illumina sequencing primer Read2: CATS paired-end sequencing primer (for read2) (C17011050) as stand-alone (*)	1-5
NextSeq 500	Illumina standard sequencing primer for read1	Read1: Illumina sequencing primer Read2: CATS paired-end sequencing primer (for read2) (C17011050) as stand-alone (*)	20
HiSeq 3000/4000	Illumina standard sequencing primer for read1	Read1: Illumina sequencing primer Read2: CATS paired-end sequencing primer (for read2) (C17011050) as stand-alone (*)	20

(*) 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 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

Short protocol for experienced users

1. Add 1 µl of RNA fragmentation buffer to the 7 µl of the template RNA (10 ng – 100 pg)
2. Incubate at 94°C during 7 minutes (RIN > 8) on a thermal cycler (lid=105°C). If the template RNA is degraded (RIN <8), please perform a fragmentation time course to find the optimal fragmentation conditions.
3. Meanwhile, prepare dephosphorylation master mix (DMM) by mixing 2 µL Dephosphorylation buffer (DB) (yellow cap) and 0.3 µL Dephosphorylation reagent (DR) (yellow cap).
4. Add 2 µL DMM to 8 µl of template fragmented RNA. Mix by pipetting up and down 5 times. Unused DMM can be stored at -20°C and thawed up to 3 times.
5. Incubate 15 min at 37°C on thermal cycler (lid=105°C).
6. Meanwhile, prepare tailing master mix (TMM) by mixing 1 µL of Tailing buffer PLUS (TBP) (red cap) and 0.5 µL Tailing Reagent PLUS (TRP) (red cap).
7. Add 1.5 µL TMM to the reaction tube. Mix by pipetting up and down 5 times.
8. Centrifuge tubes shortly (1-2 sec) at max 16.000g – **VERY IMPORTANT**
9. Incubate 40 min at 37°C + 20 min at 65° on thermal cycler (lid=105°C). Then, the sample can be kept on ice afterwards.
10. Add (purple cap)

    1 µl RT primer H (RTPH) for **10 ng – 500 pg** RNA

    or 1 µL RT primer M (RTPM) for **500 pg – 100 pg** RNA

    NO NEED TO MIX.
11. Incubate 2 min at 72°C + 2 min at 42°C on thermal cycler (lid=105°C).
12. Add 6 µL Reverse Transcription Reagent (RTR) (purple cap) and mix by pipetting up and down 5 times.
13. Incubate on thermal cycler at 42°C for 15 min (lid=105°C).
14. Add 2 µL Template Switching Reagent (TSR) (purple cap) and mix by pipetting up and down 5 times.
15. Incubate 120 min at 42°C + 10 min at 70°C on thermal cycler (lid=105°C).
    After reverse transcription, the sample can be kept at +4°C overnight (lid=25°C) or frozen until further use.
16. 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).
17. PCR reaction: (lid=105°C)
    Initial denaturation: 98°C for 30 sec

    Amplification cycles: **+ from 10** (10 ng RNA input) **to 16** (100 pg RNA input) cycles.

    Cycle:
    (denaturation) 98°C for 10 sec
    (hybridization) 62°C for 30 sec
    (elongation) 72°C for 30 sec

    Final extension: 72°C for 10 min.
    Hold at +4°C (lid=25°C) or store at -20°C until further use
18. Clean up the library with 0.9X AMPure® XP beads (90 µl) and elute the product in 50 µl of water
19. Repeat the 0.9X clean-up procedure (45µl) with AMPure® XP beads but elute the purified library in 20 µl 1X TE buffer.
20. Perform a quality check of the library with Qubit® + BioAnalyzer®
21. Load to HiSeq or MiSeq
    For paired-end sequencing, use Read2 Custom-seq primer

Detailed protocol

Notice before starting:

The protocol has been developed for inputs ranging from 10 ng to 100 pg of RNA. A starting sample volume of 7 µl is used in the library preparation process. If the volume is lower, please fill to 7 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.

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

Starting material:

- **a.** 10 ng – 100 pg isolated poly(A) mRNA : CATS mRNA-seq Kit
- **b.** 10 ng – 100 pg rRNA depleted RNA : CATS Total RNA-seq Kit
- **c.** 10 ng – 100 pg total RNA : CATS RNA-seq Kit (without sample preparation module)

1. RNA fragmentation

1. Add 1 µl of RNA fragmentation buffer to the 7 µl of template RNA (10 ng – 100pg) and mix by pipetting up and down 5 times.
2. Incubate at 94°C for 7 min (lid=105°C). (RIN >8) and then immediately cool the sample on ice. *If the template RNA is degraded (RIN <8), please perform a fragmentation time course to find the optimal fragmentation condition.*

2. RNA de-phosphorylation and tailing

3. Prepare RNA dephosphorylation master mix (DMM) by mixing 2 µL Dephosphorylation buffer (DB) (yellow cap) and 0.3 µL Dephosphorylation reagent (yellow cap) in a sterile nuclease-free PCR tube.
4. Mix 8 µL fragmented 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.
5. Incubate 15 min at 37°C on thermal cycler (lid=105°C).
6. Meanwhile, prepare RNA tailing master mix (TMM) by mixing 1 µL Tailing buffer PLUS (TBP) (red cap) with 0.5 µL Tailing Reagent PLUS (TRP) (red cap) in a sterile nuclease-free PCR tube.
7. Add 1.5 µL TMM to the reaction tube and mix by pipetting up and down 5 times.
8. Centrifuge tubes for a short time (1-2 sec) at max speed (> 10.000g).
9. Incubate 40 min at 37°C + 20 min at 65°C on thermal cycler (lid=105°C).

3. Reverse transcription

10. Add (purple cap)
    1 µl RT primer H (RTPH) for **10 ng – 500 pg** RNA
    or 1 µL RT primer M (RTPM) for **500 pg – 100 pg** RNA
    NO NEED TO MIX.

11. Incubate 2 min at 72°C + 2 min at 42°C on thermal cycler (lid=105°C).
12. Add 6 µL Reverse Transcription Reagent (RTR) (purple cap) and mix by pipetting up and down 5 times.
13. Incubate on thermal cycler at 42°C for 15 min (lid=105°C).
14. Add 2 µL Template Switching Reagent (TSR) (purple cap) and mix by pipetting up and down 5 times.
15. Incubate 120 min at 42°C + 10 min at 70°C on thermal cycler (lid=105°C).
    After reverse transcription, the sample can be kept at +4°C overnight (lid=25°C) or frozen until further use.

4. PCR pre-amplification and purification

16. 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).
17. Place the tube in a thermal cycler with a heated lid (105°C) and run the following program:

98°C for 30 sec
10 sec at 98°C
30 sec at 62°C
30 sec at 72°C
] 10 - 16 cycles (consult the table)
10 min at 72°C
Hold at +4°C (lid=25°C) or store at -20°C until further use.

Input RNA	Cycles
10 ng	10
1 ng	14
100 pg	16

18. Clean up the library with 0.9X AMPure® XP beads to remove small DNA fragments
    - Let the beads warm to room temperature on a rotating wheel or shaking device
    - Add 90 µL of AMPure® XP beads to the 100 µL of sample and mix thoroughly by pipetting up and down several times or by vortexing a few seconds
    - Incubate at room temperature for 5 min on a rotating wheel
    - Pulse spin the tube and put it on a magnetic rack until the beads have collected on the side of the tube
    - Discard the supernatant once it is completely clear
    - Wash the sample with 200 µL of 80% ethanol for 30 sec without disturbing the beads pellet
    - Take out the supernatant without disturbing the beads pellet and repeat the wash step once again
    - Pulse spin the tube and put it on a magnetic rack to attract the beads on the wall of the tube
    - Evacuate the residual ethanol by pipetting
    - Air dry the beads pellet at room temperature with the tube cap opened for 2-3 minutes
    - Resuspend the beads pellet in 50 µL of water
    - Wait for 2 minutes, during which time the DNA is solubilized
    - Put the tube on the magnetic rack and wait for the beads to be attracted completely

- Transfer the clear supernatant in a sterile nuclease-free 1.5 ml tube
- Repeat the clean-up procedure with 0.9X AMPure® XP beads (45 µL) but elute the purified library in 20 µL of 1X TE buffer.

4. Quality control of the library

19. Measure the concentration of the DNA in the purified library with a Qubit® dsDNA HS Assay Kit

20. Load 1 µl of the purified library 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.

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 20 µL eluate after AMPure® XP beads clean-up should be typically between 150 – 400 ng when quantified with 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.

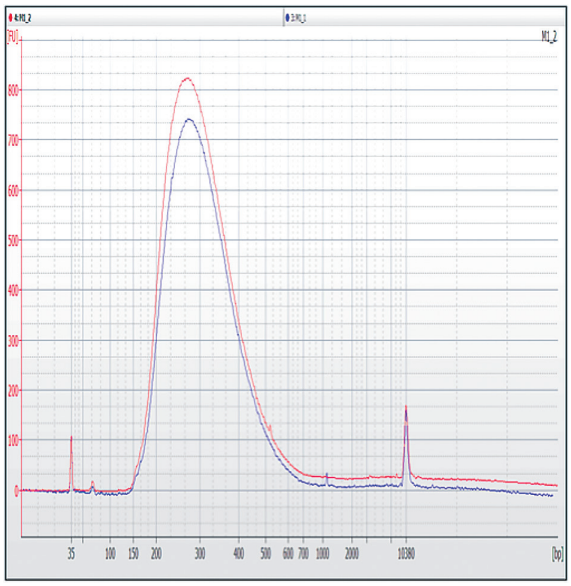

Figure 6: BioAnalyzer® DNA electrophoregram of CATS library made from 1 ng of rRNA depleted RNA isolated from total human universal RNA (Agilent, 740000)

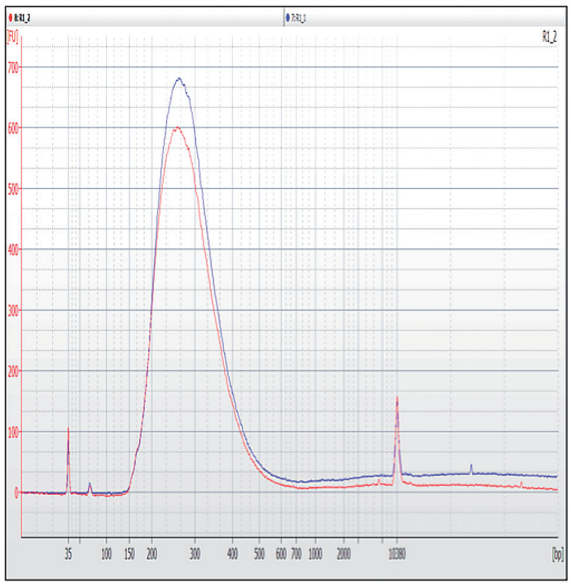

Figure 5: BioAnalyzer® DNA electrophoregram of CATS library made from 1 ng of poly(A) mRNA isolated from total human universal RNA (Agilent, 740000)

References

Turchinovich A, Surowy H, Burwinkel. "Synthesis of double-stranded nucleic acids" // application number: EP14168313.6; submission number: 2761687; date of receipt: 14 May 2014; submitted by: CN=Thomas Böhmer 36692, ZSP Patentanwälte.

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
CATS mRNA-seq Kit (with polyA selection) v2	C05010047 C05010043 C05010051	12 rxns 24 rxns 96 rxns
CATS RNA-seq Kit v2	C05010045 C05010041 C05010049	12 rxns 24 rxns 96 rxns
CATS paired-end sequencing primer	C17011050	50 µl / 500 µl

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Innovating Epigenetics Solutions

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