diagenode

A Hologic Company

CATS Total RNA-seq Kit v2 (with rRNA depletion)

CATS Total RNA sequencing kit for Illumina®

Cat. No. **C05010046** (12 rxns)
C05010042 (24 rxns)
C05010050 (96 rxns)

Version 2 I 09.17

Content

A. rRNA DEPLETION

B. CATS LIBRARY PREPARATION

MANUAL

A. rRNA depletion

Introduction

The Diagenode's rRNA depletion module depletes both cytoplasmic (5S rRNA, 5.8S rRNA, 18S rRNA, 28S rRNA) and mitochondrial ribosomal RNA (12S rRNA and 16S rRNA) from human, mouse and rat total RNA samples. This product is suitable for both intact and degraded RNA preparations (e.g. FFPE tissue). The resulting rRNA depleted RNA is recommended to be used in combination with the CATS Total RNA-seq kit.

Kit method overview & time table

Input amount	Time				Workflow time
	RNA/probe hybridization	RNase H digestion	DNase I digestion	Clean-up	
10 ng – 1µg total RNA	Hands on				
	2 min.	2 min.	2 min.	2 min.	8 min
	Total				
	22 min.	32 min.	32 min.	27 min.	1h53min

Kit materials

Description	Quantity (12 rxns)	Quantity (24 rxns)	Quantity (96 rxns)	Storage
RNase H	24 µL	48 µL	192 µL	-20°C
RNase H Reaction Buffer	24 µL	48 µL	192 µL	-20°C
rRNA Depletion Solution	12 µL	24 µL	96 µL	-20°C
Probe Hybridization Buffer	24 µL	48 µL	192 µL	-20°C
DNase I (RNase-free)	30 µL	60 µL	240 µL	-20°C
DNase I Reaction Buffer	60 µL	120 µL	480 µL	-20°C
Nuclease-free water	282 µL	1.5 ml	6 ml	-20°C

Required materials not provided

General:

- Gloves
- Single channel pipettes and corresponding RNase-free **filter** tips: 10 µL, 20 µL, 200 µL, 1,000 µL
- Crushed ice
- RNase AWAY™ decontamination reagent (ThermoFisher Scientific, 10328011)
- Table top centrifuge with strip rotor
- Tube holder for 0.2 ml tubes
- DiaMag 0.2 ml tube magnetic rack (Diagenode, B04000001)
- Thermal cycler
- Agencourt RNAClean XP beads (Beckman Coulter Inc., #A63987)

Remarks before starting

The RNA sample should be free of salts (Mg2+, guanidinium salts) or organic compounds (phenol, ethanol).

The actual yield of the process is dependent on the quality of the input RNA, the rRNA content of the sample and the method used to purify the total RNA. Typical recoveries are in between 3 to 10% of the starting amount of total RNA.

We recommend whenever possible to use inputs higher than 10 ng in order to increase the final library complexity and reduce sequencing duplication rate.

Protocol

Starting material: 10ng – 1µg of total RNA in a 12µL total volume.

1. Hybridize the probe to the RNA

1. Prepare a RNA/probe master mix as follows:

Component	Volume
rRNA Depletion Solution	1µL
Probe Hybridization Buffer	2µL
Total Volume	3µL

2. Add 3µL of the above mix to 12µL total RNA sample.
3. Mix by pipetting up and down.
4. Spin down briefly in a tabletop centrifuge, and immediately proceed to next step.
5. Place samples in a thermocycler, and run the following program which will take approximatively 15-20 minutes to complete:

Temperature	Time
95°C	2 min.
95-22°C	0.1°C/sec.
22°C	5 min. hold

6. Spin down the samples in a table top centrifuge and place on ice. Proceed immediately to the next step.

2. RNase H digestion

7. On ice, prepare a master mix according to the following table, and mix by pipetting up and down. Use immediately.

Component	Volume
RNase H	2µL
RNase H Reaction Buffer	2µL
Nuclease-free Water	1µL
Total Volume	5µL

8. Add 5µL of the above mix to the RNA sample from step 6.
9. Mix by pipetting up and down.
10. Place the samples in a thermocycler (lid = 40°C) and incubate at 37°C for 30 minutes.
11. Spin down the samples in a table top centrifuge and place on ice. Proceed immediately to the next step tp prevent non-specific degradation of RNA.

3. DNase I digestion

12. On ice, prepare a DNase I Digestion Master Mix according to the following table and mix by pipetting and down. Use immediately.

Component	Volume
DNase I Reaction Buffer	5µL
DNase I (RNase-free)	2.5µL
Nuclease-free Water	22.5µL
Total Volume	30µL

13. Add 30µL of the above mix to the RNA sample from step 11 and mix by pipetting up and down.
14. Place samples in a thermocycler (lid = 40°C) and incubate at 37°C for 30 minutes.
15. Spin down the samples in a tabletop centrifuge and place on ice. Proceed immediately to the next step.

4. RNA purification after rRNA depletion

16. Add 2.2X (110µL) Agencourt RNAClean XP beads to the RNA sample from step 15 and mix by pipetting up and down.
17. Incubate samples on ice for 15 minutes.
18. Place the tube on an appropriate magnetic rack to separate the beads from the supernatant.
19. When the solution is clear, discard the supernatant.
20. Add 200 µL of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds and then carefully remove and discard the supernatant.
21. Repeat step 20 once for a total of 2 washes.
22. Briefly spin the tube and put the tube back in the magnetic rack.
23. Completely remove the residual ethanol and air dry the beads.
24. Remove the tube from the magnetic rack. Elute RNA from the beads with 8µL of nuclease-free water.
25. Mix well by pipetting up and down and put the tube in the magnetic rack until the solution is clear.
26. Transfer 6µL of the supernatant to a clean PCR tube.
27. Assess the yield and size distribution of the sample using the BioAnalyzer® 2100 instrument with the RNA 6000 Pico kit.
    Typically, 10-20% of the starting sample amount is recovered after rRNA depletion using this module.
28. Place the sample on ice and proceed with CATS library preparation kit or, alternatively, store the sample at -80°C until further use.

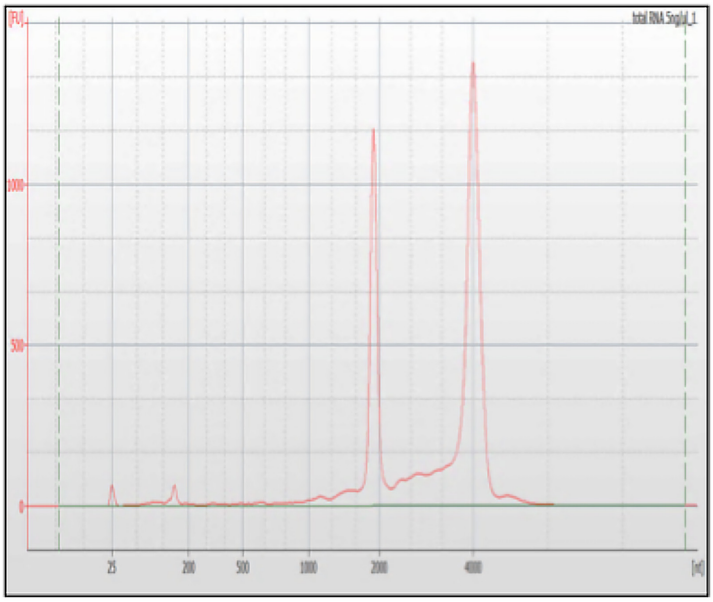

Figure 1: BioAnalyzer® RNA electrophoregram of total RNA. Starting material of the rRNA depletion

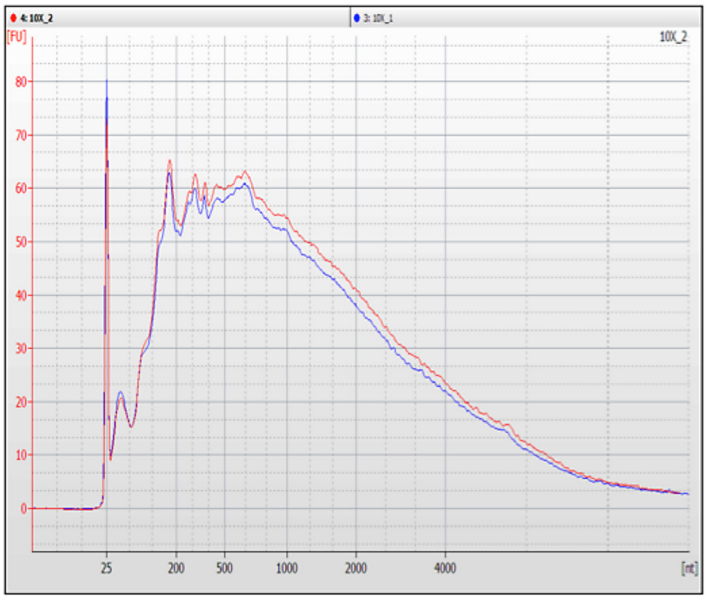

Figure 2: BioAnalyzer® RNA electrophoregram of rRNA depleted RNA. Two technical replicates

B) CATS library preparation

Introduction

The 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 a rRNA depletion module. The CATS procedure incorporates Illumina®-specific adapters in a highly efficient ligation-independent manner and avoids biases associated with adapter ligation.

The CATS 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 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.

The 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

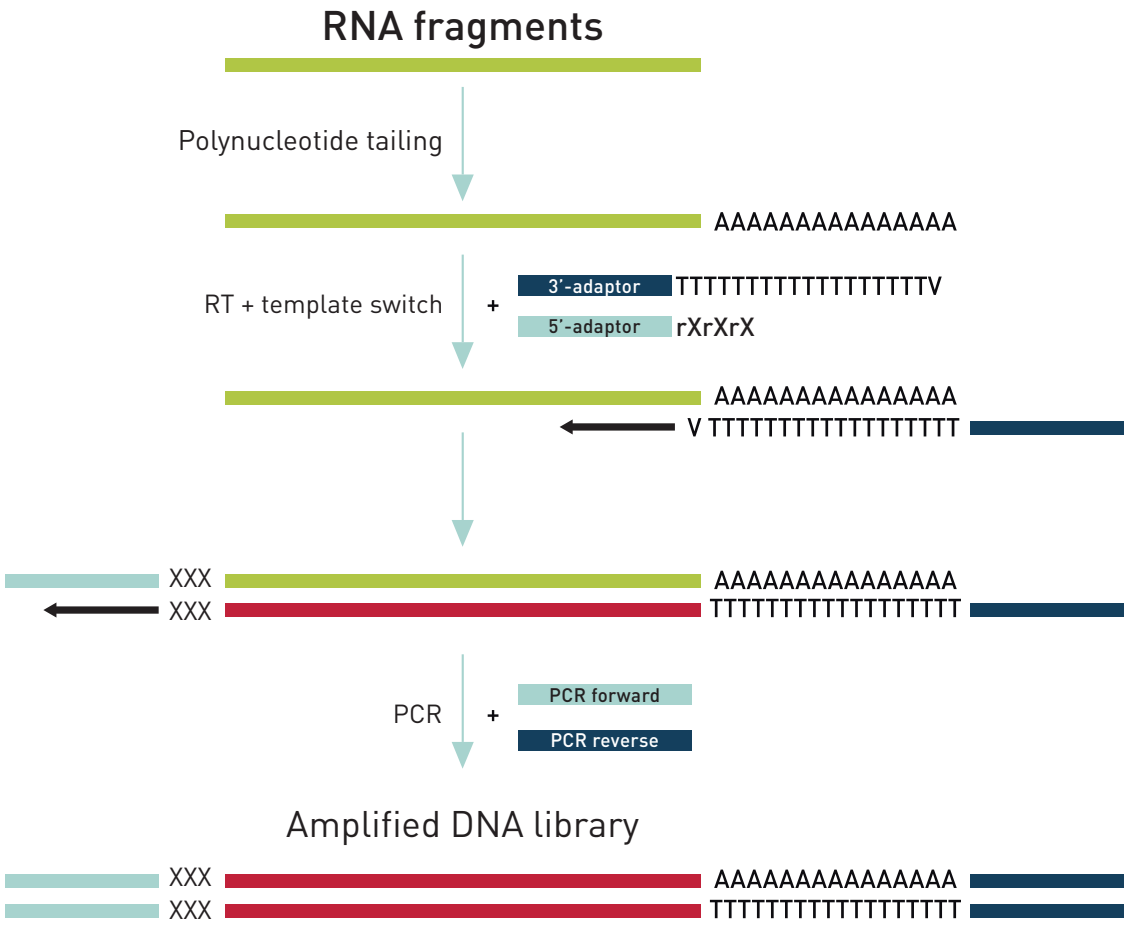

Figure 3: Schematic representation of the workflow used by the CATS RNA-seq Kit. Single stranded RNAs are first chemically fragmented, end-repaired and polyadenylated at the 3'-end. Subsequently, a cDNA strand synthesis is performed in the presence of the anchored poly(dT) oligonucleotide containing terminal P7 Illumina® adaptor sequence. When reverse transcriptase reaches the 5'-end of the RNA, it switches the template and continues DNA synthesis over the template switching oligonucleotide (TSO). The TSO contains three 3'-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 multiplexing of libraries are 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: 10µL, 20µL, 200µL, 1000µL
- 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 at the 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 control included in the CATS kit.

Sample requirements

Template

The CATS library preparation method has been developed with high quality, intact and RNA template which 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 is also expected to work 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 should 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 is offered in 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 included poly(A) mRNA isolation module. If whole transcriptome sequencing is of interest, we recommend the CATS total RNA-seq kit (Cat. C05010042) with 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 substitution to 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 1X TE buffer.

The CATS library preparation kit has been developed with input 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

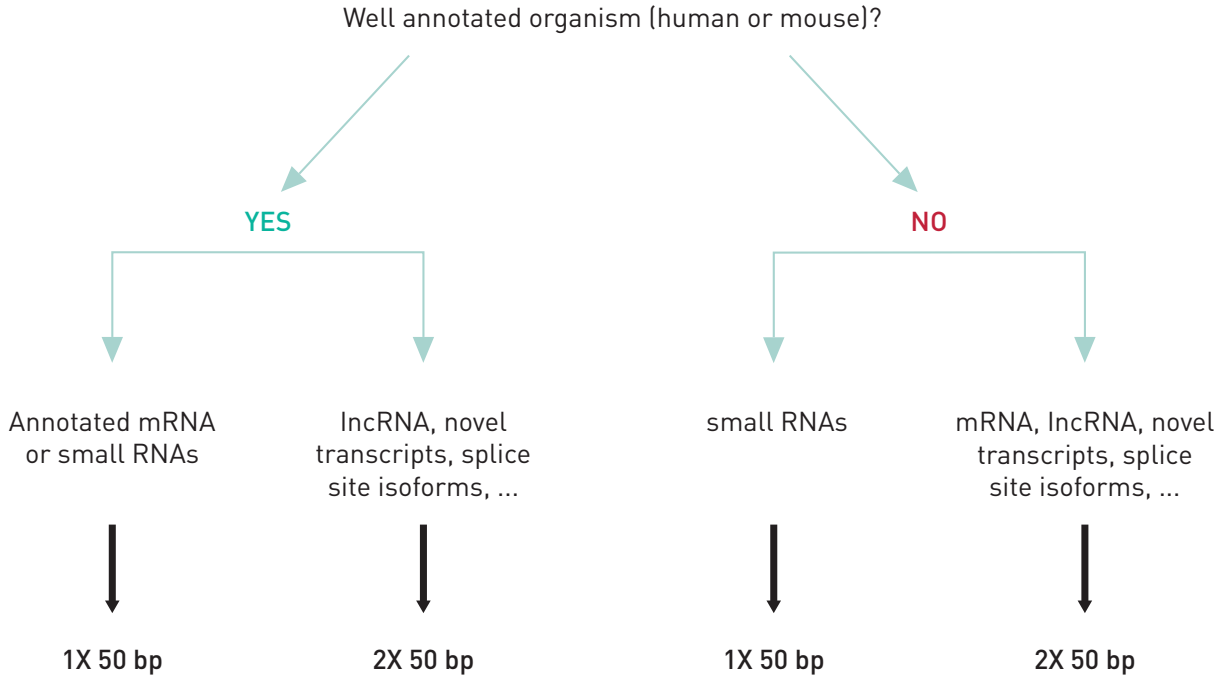

Figure 4: sequencing recommendations for Illumina® platform

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 total RNA-seq 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 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

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 thermal cycler (lid=105°C). If the template RNA is degraded (RIN < 8), please perform a fragmentation time course to find the optimal fragmentation condition.
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 or frozen until further use.
    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 ® (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 total 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 (45 µL) with AMPure® XP beads but elute the purified library in 20 µL 1X TE buffer.
20. Perform 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 input 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 conditions.

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 (lid=25°C) overnight 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 below)
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.

5. Quality control of the library

19. Measure the concentration of the DNA in the purified library with 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 should be anticipated:

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 primers (40-80 bp) or/and "empty" DNA libraries (~143 bp) should not exceed 5-10% of the total DNA libraries.

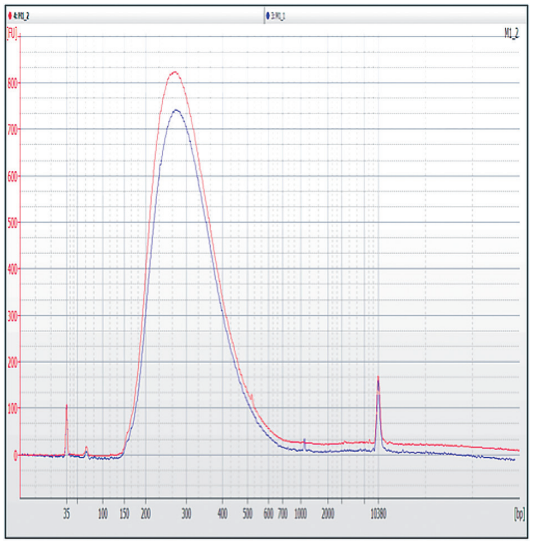

Figure 3: BioAnalyzer® DNA electrophoregram of CATS library made from 1 ng of poly(A) mRNA isolated from total human universal RNA (Agilent, 740000)

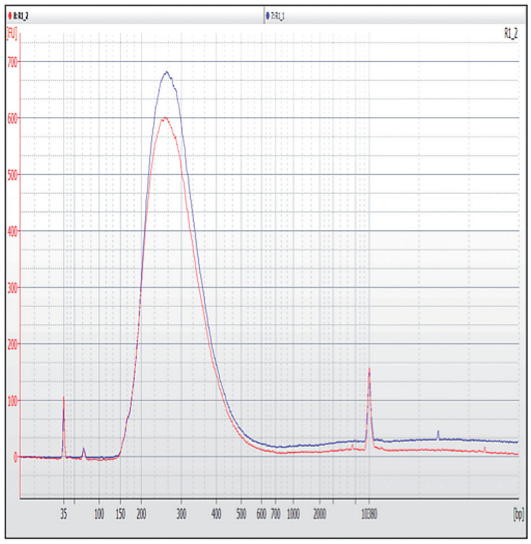

Figure 4: BioAnalyzer® DNA electrophoregram of CATS library made from 1 ng of rRNA depleted RNA isolated from total human universal RNA (Agilent, 740000)

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 Small RNA-seq Kit	C05010044 C05010040 C05010048	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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