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Hiseq 2000 Library Construction and Sequencing for RNA Seq

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1 *Works for me* dx.doi.org/10.17504/protocols.io.38kgruw

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ABSTRACT

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THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0050226

STEPS MATERIALS

NAME	CATALOG #	VENDOR
DNaseI		NEB
Dynabeads mRNA purification kit		Life Technologies
fragmentation buffer		Life Technologies
SuperScript II reverse transcription kit		Life Technologies
RNase H		Life Technologies
DNA polymerase		Enzymatics
QIAquick PCR purification kit		Qiagen
Agencourt AMPure beads		Beckman Coulter
T4 DNA polymerase and T4 polynucleotide kinase		Enzymatics
Klenow (3' to 5' exo-)		Enzymatics
dATP		Ge Healthcare
Phusion DNA polymerase		NEB

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1 Isolate polyA RNA from 20 µg of total RNA treated by

DNaseI
by NEB

using

Dynabeads mRNA purification kit
by Life Technologies

.It is best to use up to 50 µg as the use of a lower mass (typically 20 µg) has been insufficient for successful library construction. This can be assessed by running final PCR products on an agarose gel; the library construction is considered to have failed when there was no visible band. It is possible to use less than 20 µg of total RNA when isolation of an important sample yielded low RNA mass but library construction was successful.

2 Purified polyA RNA is fragmented in a

fragmentation buffer
by Life Technologies

at 70 °C for 00:01:30 to 200–300 nt fragment sizes.

3 The first cDNA strand is then synthesized with random hexamer primers using the

SuperScript II reverse transcription kit
by Life Technologies

.

4 The second-strand synthesis is performed by incubation with

RNase H
by Life Technologies

and

DNA polymerase
by Enzymatics

.

5 Short double-stranded cDNA fragments are then purified using one of two methods.

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6 Our standard procedure was to use the

QIAquick PCR purification kit
by Qiagen

, whereas for samples with low RNA mass it is better to use

Agencourt AMPure beads
by Beckman Coulter

.

7 Both methods are then followed by end-repair with Klenow polymerase,

T4 DNA polymerase and T4 polynucleotide kinase
by Enzymatics

.

8 A single 3' adenosine (A base) was added to the double-stranded cDNA using

Klenow (3' to 5' exo-)
by Enzymatics

and

dATP
by Ge Healthcare

.

9 The Illumina PE Adapter oligo mix is ligated onto the A base on repaired double-stranded cDNA ends and DNA fragments of a selected size are then gel-purified to make sure the insert size is 200 bp (±10% deviation).

10 Thereafter, libraries were amplified by 15 cycles of PCR with

Phusion DNA polymerase
by NEB

and “indexed” paired-end PCR primers; the prepared libraries were 322 bp long.

11 The amplified libraries were denatured with sodium hydroxide and diluted to [M]**2.5 Picomolar (pM)** in hybridization buffer for loading into a HiSeq flowcell.

12 Read lengths viewed on the HiSeq platform were predominately 90 bp with a small number of sequences in the 84–87 bp range.

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13 Samples are sequenced with paired-end reads, and up to eleven samples can be multiplexed into a single lane of the Illumina Hiseq flow cell. With average run time of three to twelve days depending on read length.

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