

# The preseq Manual

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## Contents

<b>1</b>	<b>Quick Start</b>	<b>2</b>
<b>2</b>	<b>Installation</b>	<b>3</b>
<b>3</b>	<b>Using preseq</b>	<b>4</b>
<b>4</b>	<b>File Format</b>	<b>5</b>
<b>5</b>	<b>Detailed usage</b>	<b>6</b>
<b>6</b>	<b>lc_extrap Examples</b>	<b>8</b>
<b>7</b>	<b>preseq Application Examples</b>	<b>11</b>
<b>8</b>	<b>FAQ</b>	<b>15</b>

# 1 Quick Start

The **preseq** package is aimed at predicting the yield of distinct reads from a genomic library from an initial sequencing experiment. The estimates can then be used to examine the utility of further sequencing, optimize the sequencing depth, or to screen multiple libraries to avoid low complexity samples.

The two main programs are `c_curve` and `lc_extrap`. `c_curve` samples reads without replacement from the given mapped sequenced read file or duplicate count file to estimate the yield of the experiment and the subsampled experiments. These estimates are used to construct the complexity curve of the experiment. `lc_extrap` uses rational function approximations of Good & Toulmin's [3] non-parametric empirical Bayes estimator to predict the yield of future experiments, in essence looking into the future for hypothetical experiments. `lc_extrap` is used to predict the yield and then `c_curve` can be used to check the yield from the larger experiment.

## 2 Installation

### Download

**preseq** is available at <http://smithlab.cmb.usc.edu/software/>.

### System Requirements

**preseq** runs on Unix-type system with GNU Scientific Library (GSL), available at <http://www.gnu.org/software/gsl/>. If the input file is in BAM format, SAMTools is required, available at <http://samtools.sourceforge.net/>. If the input is a text file of counts in a single column or is in BED format, SAMTools is not required. It has been tested on Linux and Mac OS-X.

### Installation

Download the source code and decompress it with

```
$ tar -jxvf preseq.tar.bz2
```

Enter the **preseq/** directory and run

```
$ make all
```

The input file may possibly be in BAM format. If the root directory of SAMTools is \$SAMTools, instead run

```
$ make all SAMTOOLS_DIR=$SAMTools
```

Output after typing this command should include the flag `-DHAVE_SAMTOOLS` if the linking is successful. If compiled successfully, the executable file is available in **preseq/**.

If a BAM file is used as input without first having run `$ make all SAMTOOLS_DIR=/loc/of/SAMTools`, then the following error will occur: `terminate called after throwing an instance of 'std::string'`.

### 3 Using preseq

#### Basic usage

To generate the complexity plot of a genomic library from a read file in BED or BAM format or a duplicate count file, use the function `c_curve`. Use `-o` to specify the output name.

```
$ ./preseq c_curve -o complexity_output.txt input.bed
```

To estimate the future yield of a genomic library using an initial experiment in BED format, use the function `lc_extrap`. The required options are `-o` to specify the output of the yield estimates and the input file, which is either a BED file sorted by chromosome, end position, start position, and strand or a BAM file sorted with the SAMTools sort function. Additional options are available and are detailed below.

```
$ ./preseq lc_extrap -o future_yield.txt input.txt
```

## 4 File Format

### Sorted read files in BED or BAM format

Input files are sorted mapped read files in BED or BAM format, or a text file consisting of one column giving the observed read counts. The programs require that BED files are sorted by chromosome, end position, start position, and strand. This can be achieved by using the command line function sort as follows:

```
sort -k 1,1 -k 2,2n -k 3,3n -k 6,6 input.bed > input.sort.bed
```

BAM format read files should be sorted by chromosome and start position. This can be done with the SAMTools sort function. If the input is in BAM format, then the flag `-B` must be included.

If the input is paired end, the option `-P` can be set. In this case concordantly mapped reads and discordantly mapped fragments are counted. This means that both ends of a discordantly mapped read will each be counted separately. If a large number of reads are discordant, then the default single end should be used. In this case only the mapping location of the first mate is used as the unique molecular identifier [4].

### Text files of observed read counts

For more general applications **preseq** allows the input to be a text file of observed read counts, one count per line. To specify this input, the option `-V` must be set.

Such a text file can typically be constructed by command line arguments. Take for example an unmapped sequencing experiment in FASTQ format. To predict the complexity, the unique molecular identifier needs to use only the observed sequence. For instance, a unique molecular identifier used may be the first 20 bases in the observed sequence. A command line argument to construct the counts would then be

```
awk '{ if (NR%4==2) print substr($0,1,20); }' input.fastq | sort | uniq -c  
  
| awk '{ print $1 }' > counts.txt
```

More complicated unique molecular identifiers can be used, such as mapping position plus a random barcode, but are too complicated to detail in this manual. For questions with such usage, please contact us at [tdaley@usc.edu](mailto:tdaley@usc.edu)

## 5 Detailed usage

### **c\_curve**

`c_curve` is used to compute the expected complexity curve of a mapped read file by subsampling smaller experiments without replacement and counting the distinct reads. Output is a text file with two columns. The first gives the total number of reads and the second the corresponding number of distinct reads.

<b>-o, -output</b>	Name of output file. Default prints to screen
<b>-s, -step</b>	The step size for samples. Default is 1 million reads
<b>-v -verbose</b>	Prints more information
<b>-B, -bam</b>	Input file is in BAM format
<b>-P, -pe</b>	Input is a paired end read file
<b>-H, -hist</b>	Input is a text file of the observed histogram
<b>-V, -vals</b>	Input is a text file of read counts

### **lc\_extrap**

`lc_extrap` is used to generate the expected yield for theoretical larger experiments and bounds on the number of distinct reads in the library and the associated confidence intervals, which is computed by bootstrapping the observed duplicate counts histogram. Output is a text file with four columns. The first is the total number of reads, second gives the corresponding average expected number of distinct reads, and the third and fourth give the lower and upper limits of the confidence interval. Specifying verbose will print out the counts histogram of the input file.

<b>-o, -output</b>	Name of output file. Default prints to screen
<b>-e, -extrap</b>	Max extrapolation. Default is $1 \times 10^{10}$
<b>-s, -step</b>	The step size for samples. Default is 1 million reads
<b>-b, -bootstraps</b>	The number of bootstraps. Default is 100
<b>-c, -cval</b>	Level for confidence intervals. Default is 0.95
<b>-d, -dupl.level</b>	Fraction of duplicates to predict. Default is 0.5
<b>-x, -terms</b>	Max number of terms for extrapolation. Default is 100
<b>-v -verbose</b>	Prints more information
<b>-B, -bam</b>	Input file is in BAM format
<b>-P, -pe</b>	Input is a paired end read file
<b>-H, -hist</b>	Input is a text file of the observed histogram

**-v, -vals**

Input is a text file of read counts

**-Q, -quick**

Quick mode, option to estimate yield without bootstrapping for confidence intervals



## 6 lc\_extrap Examples

Usage and output of `c_curve` is similar, so the following examples are of `lc_extrap` and its different options.

### Using a sorted read file in BED (or BAM with the `-B` flag) format as input

```
$ ./preseq lc_extrap -o future_yield.txt input.bed
```

TOTAL_READS	EXPECTED_DISTINCT	LOGNORMAL_LOWER_95%CI	LOGNORMAL_UPPER_95%CI
0	0	0	0
1000000.0	955978.6	953946.4	958015.1
2000000.0	1897632.0	1892888.4	1902387.5
3000000.0	2829410.5	2819146.4	2839712.0
4000000.0	3751924.0	3732334.5	3771616.2
.	.	.	.
.	.	.	.
.	.	.	.
.	.	.	.
9999000000.0	185394069.4	76262245.8	450694319.0

This example uses a sorted read file in BED format from an initial experiment generated from single sperm cells. As noted above, the default step size between yield estimates is 1 million, the default confidence interval level is 95%, and the default extrapolation length is 10 billion.

### Using a sorted read file in BED format as input, including the verbose option

```
$ ./preseq lc_extrap -o future_yield.txt input.bed -v
```

As `lc_extrap` is running, information will print to screen that gives a read counts histogram of the input file which truncates after the first bin value that has zero observations. Included here is the first 10 lines of what would be observed:

```
TOTAL READS      = 536855
DISTINCT READS   = 516200
DISTINCT COUNTS  = 48
MAX COUNT        = 269
COUNTS OF 1     = 511413
MAX TERMS        = 100
OBSERVED COUNTS (270)
1      511413
2      2202
3      597
.
.
```

## Using a sorted read file in BED format as input, with options

```
$ ./preseq lc_extrap -e 15000000 -s 500000 -b 90 -c .90 -o future_yield.txt input.bed
```

TOTAL_READS	EXPECTED_DISTINCT	LOGNORMAL_LOWER_90%CI	LOGNORMAL_UPPER_90%CI
0	0	0	0
500000.0	481098.5	480329.1	481869.1
1000000.0	956070.6	954493.7	957650.2
1500000.0	1428183.4	1425461.7	1430910.2
2000000.0	1897886.0	1892501.7	1903285.7
.	.	.	.
.	.	.	.
.	.	.	.
.	.	.	.
14500000.0	12932529.0	12056525.8	13872180.9

Notice the slight changes, with the step sizes of the extrapolation now at 500,000 as specified, and the maximum extrapolations ending at 15,000,000. The confidence intervals are now at a level of 90%.

## Using a histogram or read counts as input

`lc_extrap` allows the input file to be an observed histogram. An example of the format of this histogram is as followed:

```
1      1.68166e+07
2      4.55019e+06
3      1.93787e+06
4      1.07257e+06
5      708034
6      513134
7      384077
8      282560
9      206108
10     146334
```

The following command will give output of the same format as the above examples.

```
$ ./preseq lc_extrap -o future_yield.txt -H histogram.txt
```

Similarly, both `lc_extrap` and `c_curve` allow the option to input read counts (text file should contain ONLY the observed counts in a single column). For example, if a dataset had the following counts histogram:

```
1      4
2      3
3      1
```

Then, the corresponding input file of just read counts could be as such:

```
1
2
1
1
3
2
2
1
```

Command should be run with the `-V` flag (not to be confused with `-v` for verbose mode):

```
$. /preseq lc_extrap -o future_yield.txt -V counts.txt
```

## 7 preseq Application Examples

### Screening multiple libraries

This section provides a more detailed example using data from different experiments to illustrate how preseq might be applied. Because it is important to avoid spending time on low complexity samples, it is important to decide after observing an initial experiment whether or not it is beneficial to continue with sequencing. The data in this example comes from a study (accession number SRA061610) using single cell sperm cells amplified by Multiple Annealing and Looping Based Amplification Cycles (MALBAC) [5] and focuses on three libraries coming from different experiments from the study (SRX205369, SRX205370, SRX205372).

These libraries help show what would be considered a relatively poor library and a relatively good library, as well as compare the complexity curves obtained from running `c_curve` and `lc_extrap`, to show how `lc_extrap` would help in the decision to sequence further. The black diagonal line represents an ideal library, in which every read is a distinct read (though this cannot be achieved in reality). The full experiments were down sampled at 5% to obtain a mock initial experiment of the libraries, as shown here, where we have the complexity curves of the initial experiments generated by `c_curve`:

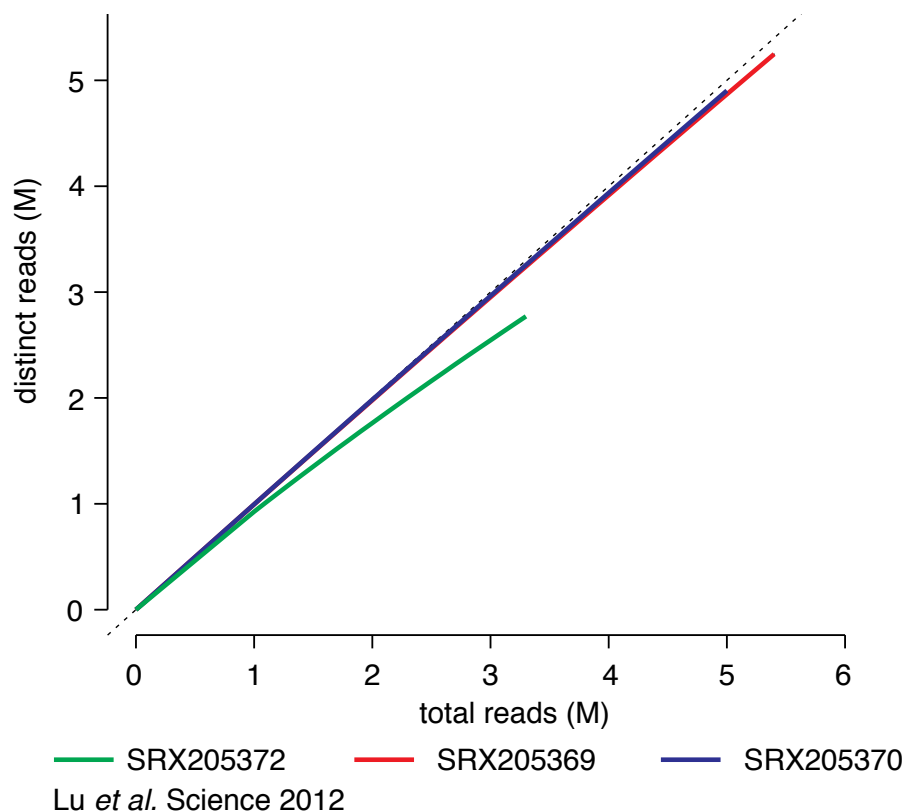


Figure 1: Initial observed complexities

With such a relatively small amount of reads sequenced, it is hard in the first stages of a study to guess at whether it is not worth sequencing a library further, as all three libraries seem to be relatively good. This is a comparison of the full experiment complexity curves and the extrapolated complexity curves created using information from the initial experiments above as input. The dashed lines indicate the complexity curves predicted by `lc_extrap`, and the solid lines are the expected complexity curves of the full experiments, obtained using `c_curve`. Note that the dashed curves follow the solid curves very closely, only differing slightly towards the end, meaning `lc_extrap` gives a good predicted yield curve. Using this, it is clear that if the initial experiments were the only available data and `lc_extrap` was run, SRX205372 would likely be discarded, as it is a poor library, and SRX205369 and SRX205370 would probably be used for further sequencing, as their complexity curves indicate that sequencing more would yield enough information to justify the costs. If the researcher were to only want to sequence one library deep, then SRX205370 would be an obvious choice.

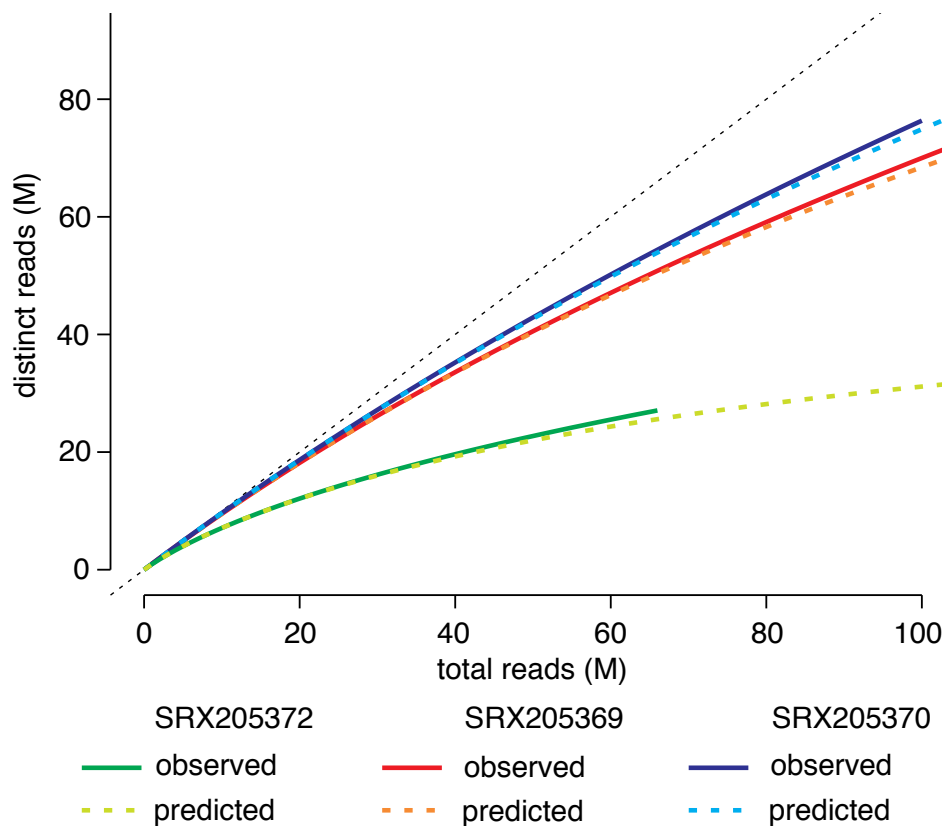


Figure 2: Estimated versus observed library complexities.

## Saturation of reads and junctions for RNA sequencing experiments

A recent paper from the Rinn lab [6] developed a targeted capture RNA sequencing protocol to deeply investigate chosen portions of the transcriptome. A comparison of the results from a standard RNA sequencing experiment (RNA-seq; SRA accession SRX061769) and a targeted capture RNA sequencing experiment (Capture-seq; SRA accession SRX061768) reveals a startling amount of transcriptional complexity missed by standard RNA sequencing in the targeted regions. A large number of rare transcriptional events such as alternative splices, alternative isoforms, and long non-coding RNAs were newly identified with the targeted sequencing.

A current vigorous debate exists on whether these rare events are truly transcriptional events or are merely due to sequencing or transcriptional noise (see [7] and [2]). We do not seek to address these issues, but merely to comment on the true complexity of rare transcriptional events in sequencing libraries identified by current protocols.

We took the two Illumina sequencing libraries from [6] and mapped them according to the protocol given. We downsampled 10% of the library and compared the estimated library complexities (single end) with the observed library complexity for both libraries. We also took the junction information contained in the file junctions.bed in the Tophat output folder to estimate the junction complexity. Since the 5th column (excluding the first line) is the number of times each distinct junction is observed, we can simply cut out these values as input for `lc_extrap` or `c_curve` with the flag `-V`. A simple command line example follows.

```
sed '1d' tophat/junctions.bed | cut -f 5 > junction_vals.txt
./preseq lc_extrap -V junction_vals.txt -o junction_vals_extrap.txt
```

The output `TOTAL_READS` column will be in terms of the number of total junctions (not reads), so scaling by the average number of junctions per read will give the appropriate scale for plotting on the x-axis.

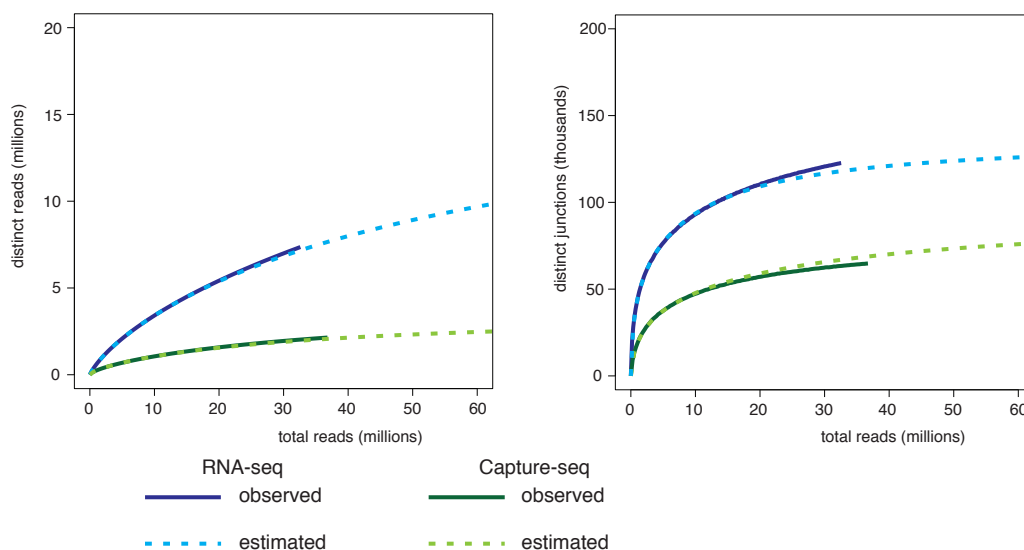


Figure 3: A comparison of complexities of standard RNA-seq and targeted capture RNA-seq. Estimated complexities for both cases were estimated using 10% of the data.

We see from the estimated library that the RNA-seq library is far from saturated, while it appears that the Capture-seq library may be close. On the other hand, the junction complexity of both libraries indicates that the full scope of junctions identified by Tophat is far from saturated in both libraries. This indicates that large number of rare junctions still remain to be identified in the libraries.

## 8 FAQ

1. Q — When compiling the preseq binary, I receive the error

```
fatal error: gsl/gsl_cdf.h: No such file or directory
```

A. — The default location of the GSL library will be in `'/usr/local/include/gsl'`. Open the Makefile and append `"-I /usr/local/include"` after `CXX = g++`. You may be receiving this error because the GSL library is not installed on the default search paths of your compiler, and you will need to specify the location.

2. Q — When compiling the preseq binary, I receive the error

```
Undefined symbols for architecture x86_64:
```

```
  "_packInt16", referenced from:
      _deflate_block in bgzf.o
  "_packInt32", referenced from:
      _deflate_block in bgzf.o
  "_unpackInt16", referenced from:
      _bgzf_read_block in bgzf.o
      _check_header in bgzf.o
```

A. — Go to the SAMTools directory and open the file `bgzf.c`. Find the functions `packInt16`, `unpackInt16`, and `packInt32`. Comment out the `"inline"` before each function name.

3. Q — I compile the preseq binary but receive the error

```
terminate called after throwing an instance of 'std::string'
```

A. — This error is typically called because either the flag `-B` was not included to specify bam input or because the linking to SAMTools was not included when compiling preseq. To ensure that the linking was done properly, check for the flag `-DHAVE_SAMTOOLS`.

4. Q — When running `lc_extrap`, I receive the error

```
ERROR: too many iterations, poor sample
```

A. — Most commonly this is due to the presence of defects in the approximation which cause the estimates to be unstable. Setting the step size larger (with the flag `-s`) will help to avoid the defects. The default step size is 1M reads or 0.05% of the input sample size rounded up to the nearest million, whichever is larger. A consequence of this action will be a reduction in the observed smoothness of the curve.

5. Q — When running `lc_extrap`, I receive the error

```
sample not sufficiently deep or duplicates removed
```

A. — There may be two causes for this, either duplicates have been removed and every observed read is distinct or there is not sufficient variation in the library for `lc_extrap` to run.



The information required by `lc_extrap` is essentially the number of times each distinct read was observed, which we call the duplicate counts. Without sufficient variation in the duplicate counts we cannot extrapolate the complexity of the library. We have set the minimum required max duplicate count (the largest number of times any read has been observed) to 4. If the input library does not satisfy this, then either a parametric model such as a Poisson or Negative Binomial may be appropriate or deeper sequencing may be required.

6. Q — When running `lc_extrap`, I receive the error

`Library expected to saturate in doubling of size, unable to extrapolate`

A. — A simple two-fold extrapolation using the Good-Toulmin power series, which is within the radius of convergence and therefore rational function approximation is not needed, is performed to ensure that the sample is not overly saturated. If the Good-Toulmin formula is negative, this indicates that the library will likely completely saturate by doubling the experiment size and so extrapolation is not needed. Often this will occur if the number of reads observed twice ( $n_2$ ) is greater than the number of reads observed once ( $n_1$ ). In this case one can use simple estimators like Chao's [1] ( $n_1^2/2n_2$ ) or Zelterman's [8] ( $1/(exp(2n_2/n_1) - 1)$ ) can be used to estimate the number of remaining in the library.

If none of these solutions worked, please email us at `tdale@usc.edu` and please include the standard output from running `preseq` in verbose mode (specifically the duplicate counts histogram) so that we can look into the problem and rectify problems in future versions. Also, feel free to email us with any other questions or concerns. The `preseq` software is still under development so we would appreciate any advice, comments, or notification of any possible bugs. Thanks!

## References

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