

基礎生物学研究所ゲノムインフォマティックス・トレーニングコース 2021夏  
RNA-seq入門：RNA-seq解析パイプライン  
2021.09.15-2021.09.16

# RNA-Seqパイプライン ゲノムベースの解析法

基礎生物学研究所  
生物機能解析センター  
山口勝司

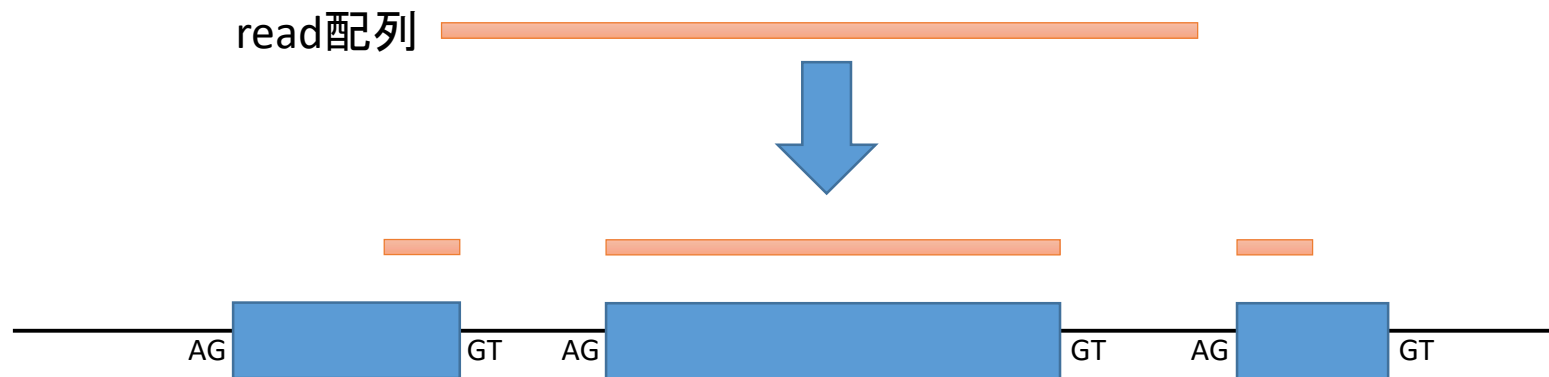
# genomeをレファレンスとする場合

---

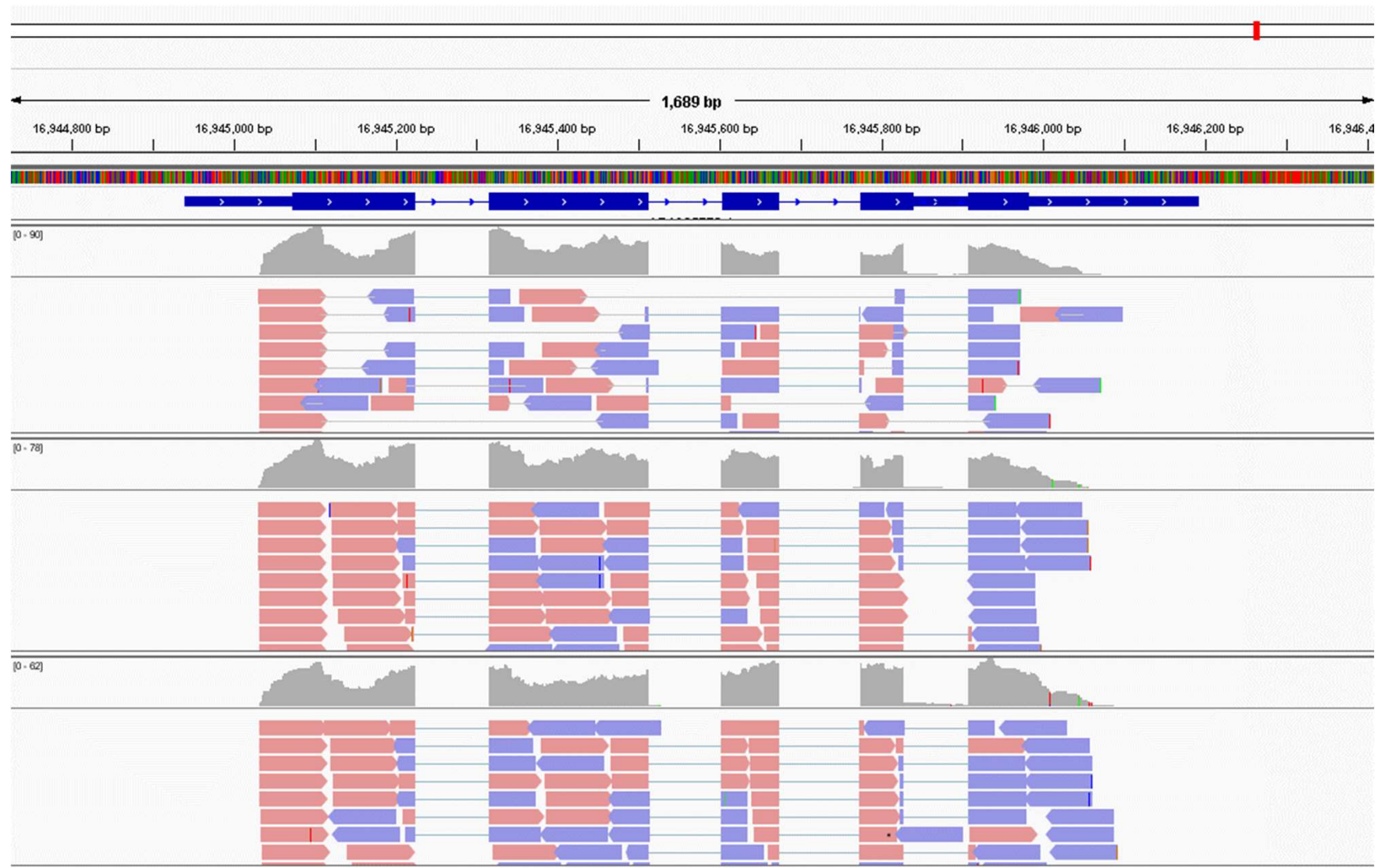
Reference配列がゲノム配列の場合、  
イントロン配列のスプライシングを考慮した  
アライメントを行う必要がある。

今回はHISAT2を用いる

他 Tophat, Blat, SpliceMap, MapSplice, GSMAP, QPALMA



# 実際こんな感じにアラインされる



# TopHat

A spliced read mapper for RNA-Seq

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CENTER FOR COMPUTATIONAL BIOLOGY  
CCB

TopHat is a fast splice junction mapper for RNA-Seq reads. It aligns RNA-Seq reads to mammalian-sized genomes using the ultra high-throughput short read aligner [Bowtie](#), and then analyzes the mapping results to identify splice junctions between exons.

TopHat is a collaborative effort among Daehwan Kim and Steven Salzberg in the [Center for Computational Biology](#) at Johns Hopkins University, and Cole Trapnell in the [Genome Sciences Department](#) at the University of Washington. TopHat was originally developed by Cole Trapnell at the [Center for Bioinformatics and Computational Biology](#) at the University of Maryland, College Park.

<https://ccb.jhu.edu/software/tophat/index.shtml>

OSI certified

» **TopHat 2.1.1 release 2/23/2016**

Please note that TopHat has entered a low maintenance, low support stage as it is now largely superseded by [HISAT2](#) which provides the same core functionality (i.e. spliced alignment of RNA-Seq reads), in a more accurate and **much more efficient** way.

Version 2.1.1 is a maintenance release which includes the following changes, some of them thanks to [GitHub](#) contributors:

Site Map

- [Home](#)
- [Getting started](#)
- [Manual](#)
- [Index and annotation downloads](#)
- [FAQ](#)

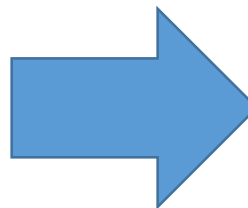
Traditional 'Tuxedo' package

New 'Tuxedo' package

TopHat



Cufflinks



HISAT

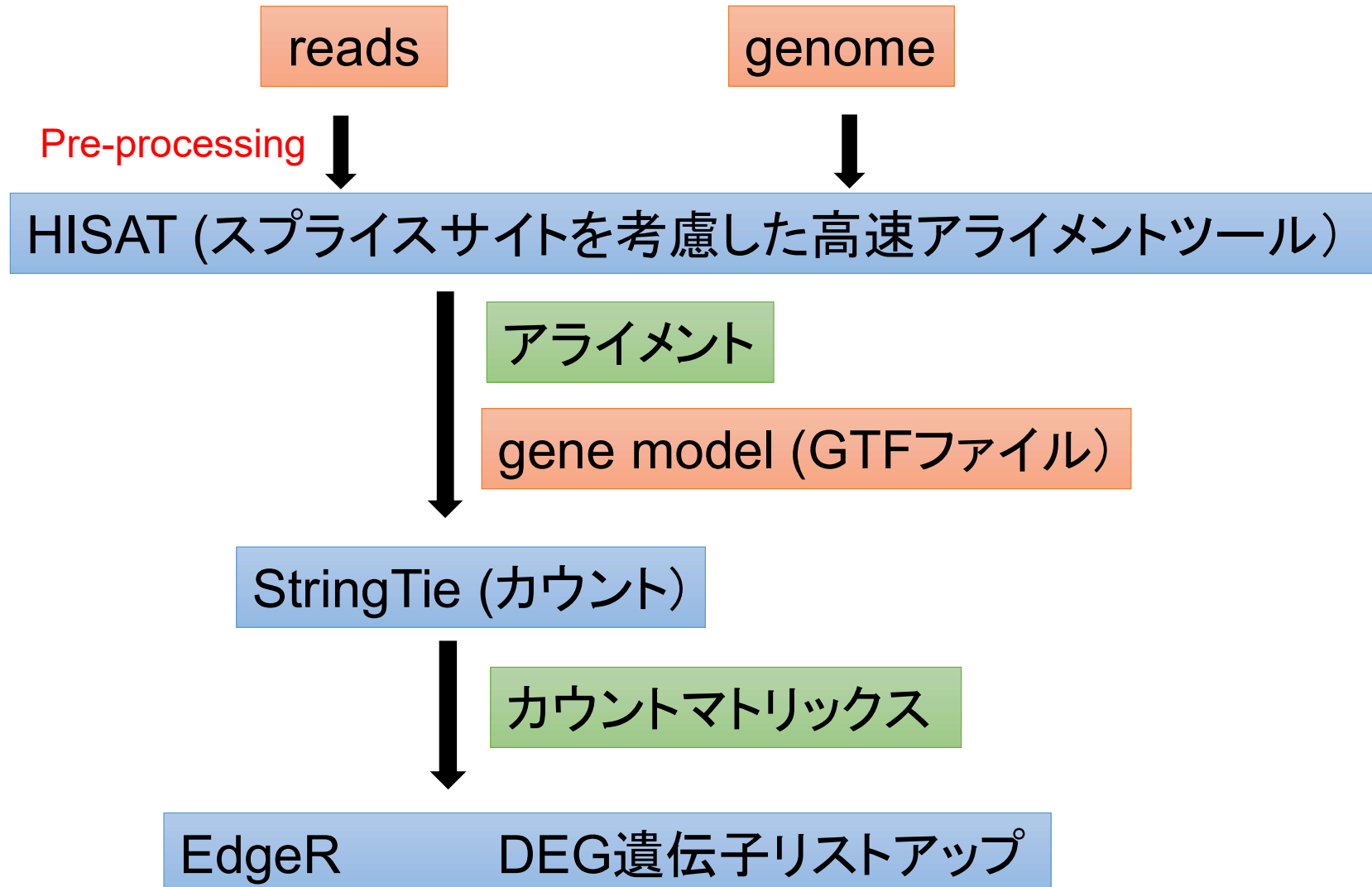


StringTie  
Ballgown

劇的に解析速度が速くなった

# 本トレーニングコースでの流れ

---



# HISAT2

**HISAT2** is a fast and sensitive alignment program for mapping next-generation sequencing reads (both DNA and RNA) to a population of human genomes as well as to a single reference genome. Based on an extension of BWT for graphs ([Sirén et al. 2014](#)), we designed and implemented a graph FM index (GFM), an original approach and its first implementation. In addition to using one global GFM index that represents a population of human genomes, **HISAT2** uses a large set of small GFM indexes that collectively cover the whole genome. These small indexes (called local indexes), combined with several alignment strategies, enable rapid and accurate alignment of sequencing reads. This new indexing scheme is called a Hierarchical Graph FM index (HGFM).

## HISAT-3N beta release 12/14/2020

HISAT-3N is a software system for analyzing nucleotide conversion sequencing reads. See the [HISAT-3N](#) for more details.

## Index files are moved to the AWS Public Dataset Program. 9/3/2020

We have moved HISAT2 index files to the AWS Public Dataset Program. See the [link](#) for more details.

## HISAT 2.2.1 release 7/24/2020

This patch version includes the following changes.

- Python3 support
- Remove the HISAT-genotype related scripts. HISAT-genotype moved to <http://daehwankimlab.github.io/hisat-genotype/>
- Fixed bugs related to `--read-lengths` option

## TopHat2と比較して速い

[Main](#)[About](#)[Manual](#)[HISAT-3N](#)[Download](#)[HowTo](#)[Links](#)

## Funding

This work was supported in part by the National Human Genome Research Institute under grants R01-HG006102 and R01-HG006677, and NIH grants R01-LM06845 and R01-GM083873 and NSF grant CCF-0347992 to Steven L. Salzberg and by the Cancer Prevention Research Institute of Texas under grant RR170068 and NIH grant R01-GM135341 to Daehwan Kim



# Manual

## Introduction

パラメータの意味など  
詳しく知るためには、  
必ずManualを見る

## What is HISAT2?

HISAT2 is a fast and sensitive alignment program for mapping next-generation sequencing reads (whole-genome, transcriptome, and exome sequencing data) against the general human population (as well as against a single reference genome). Based on [GCSA](#) (an extension of [BWT](#) for a graph), we designed and implemented a graph FM index (GFM), an original approach and its first implementation to the best of our knowledge. In addition to using one global GFM index that represents general population, HISAT2 uses a large set of small GFM indexes that collectively cover the whole genome (each index representing a genomic region of 56 Kbp, with 55,000 indexes needed to cover human population). These small indexes (called local indexes) combined with several alignment strategies enable effective alignment of sequencing reads. This new indexing scheme is called Hierarchical Graph FM index (HGFM). We have developed HISAT 2 based on the [HISAT](#) and [Bowtie2](#) implementations. HISAT2 outputs alignments in [SAM](#) format, enabling interoperability with a large number of other tools (e.g. [SAMtools](#), [GATK](#)) that use SAM. HISAT2 is distributed under the [GPLv3 license](#), and it runs on the command line under Linux, Mac OS X and Windows.

---

**PROTOCOL**

# Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown

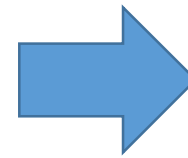
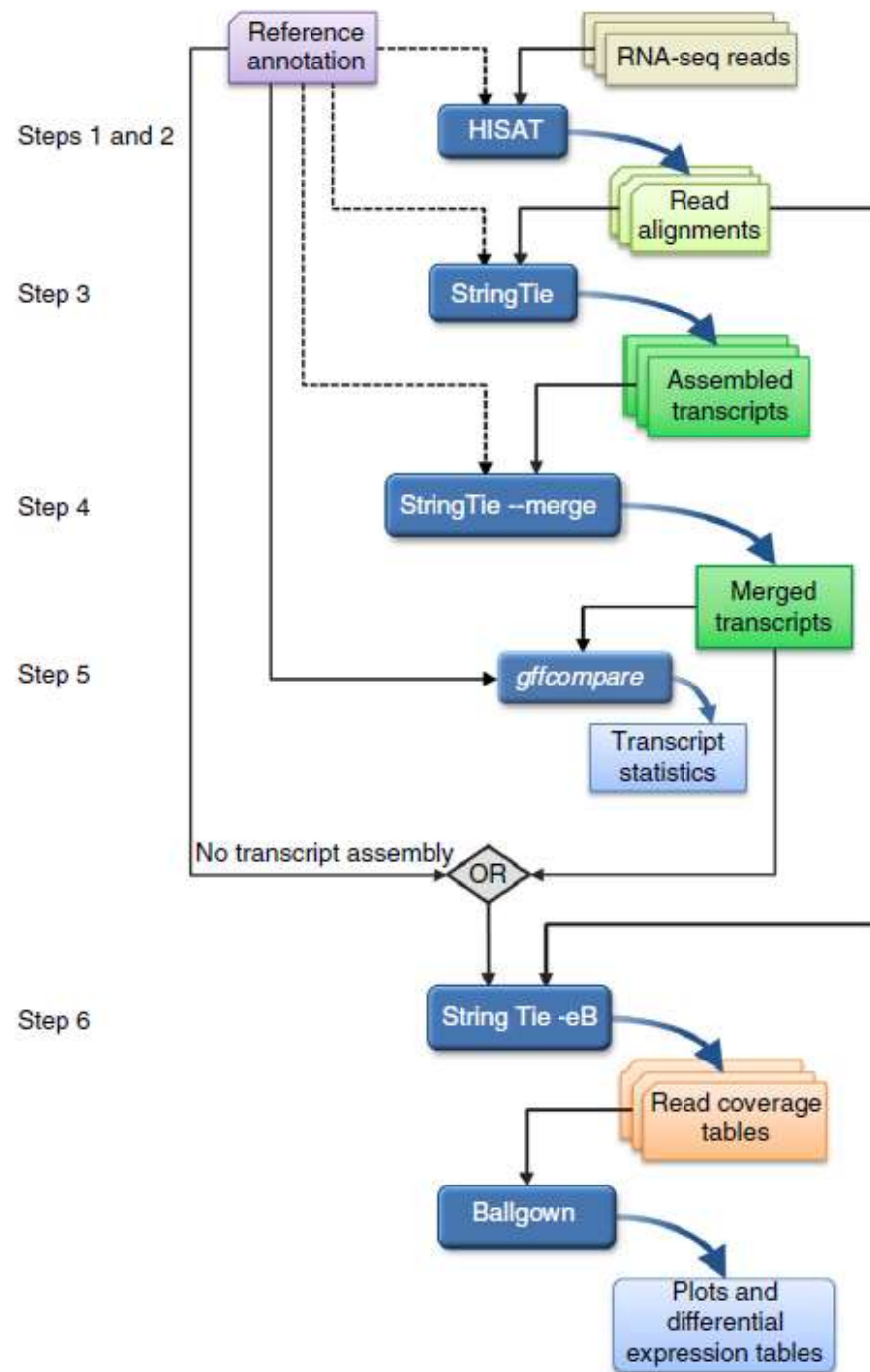
Mihaela Pertea<sup>1,2</sup>, Daehwan Kim<sup>1</sup>, Geo M Pertea<sup>1</sup>, Jeffrey T Leek<sup>3</sup> & Steven L Salzberg<sup>1–4</sup>

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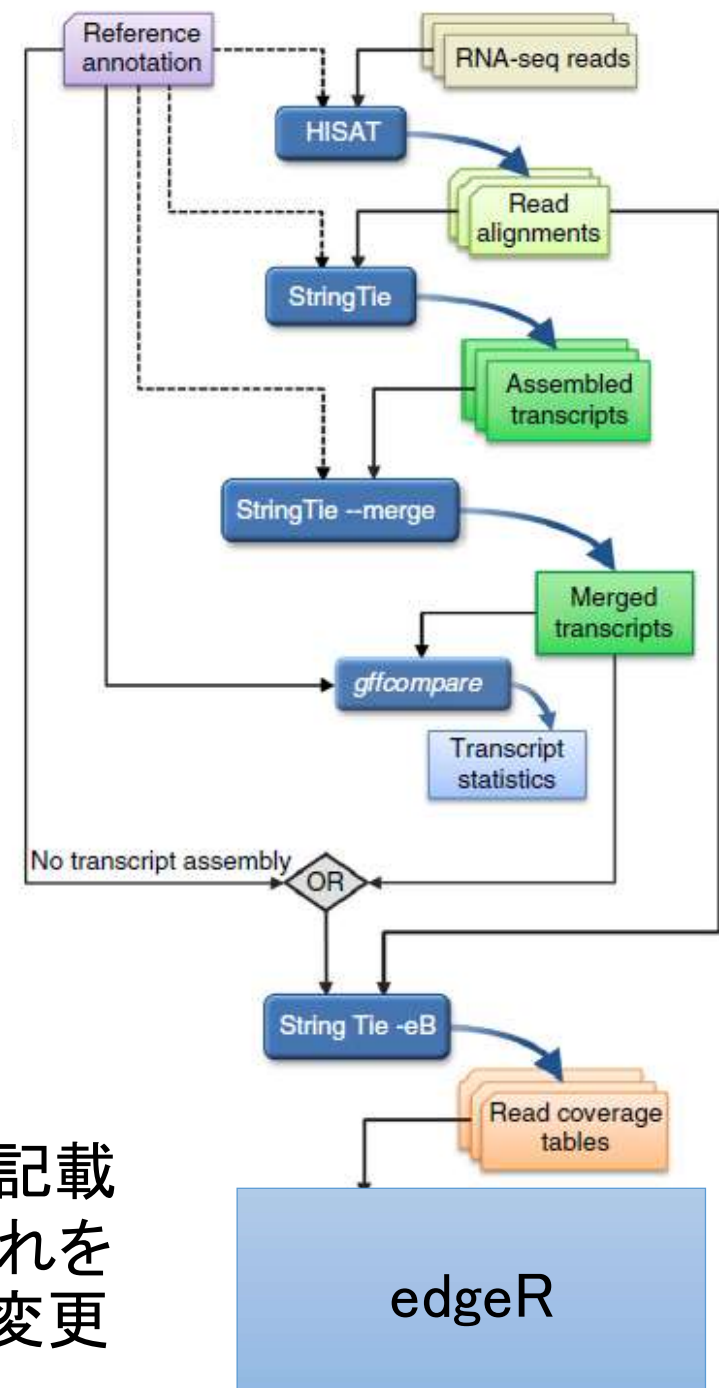
<sup>1</sup>Center for Computational Biology, McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins School of Medicine, Baltimore, Maryland, USA. <sup>2</sup>Department of Computer Science, Whiting School of Engineering, Johns Hopkins University, Baltimore, Maryland, USA. <sup>3</sup>Department of Biostatistics, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland, USA. <sup>4</sup>Department of Biomedical Engineering, Johns Hopkins University, Baltimore, Maryland, USA. Correspondence should be addressed to S.L.S. ([salzberg@jhu.edu](mailto:salzberg@jhu.edu)).

Published online 11 August 2016; doi:[10.1038/nprot.2016.095](https://doi.org/10.1038/nprot.2016.095)





論文記載  
の流れを  
少し変更



# hisat2-buildでリファレンスのインデックスを作る

---

```
$ hisat2-build -h
HISAT2 version 2.1.0 by Daehwan Kim (infphilo@gmail.com, http://www.ccb.jhu.edu/people/infphilo)
Usage: hisat2-build [options]* <reference_in> <ht2_index_base>
    reference_in          comma-separated list of files with ref sequences
    hisat2_index_base     write ht2 data to files with this dir/basename
Options:
    -c                   reference sequences given on cmd line (as
                        <reference_in>)
    --large-index        force generated index to be 'large', even if ref
                        has fewer than 4 billion nucleotides
    -a/--noauto          disable automatic -p/--bmax/--dcv memory-fitting
    -p                   number of threads
    --bmax <int>         max bucket sz for blockwise suffix-array builder
    --bmaxdivn <int>     max bucket sz as divisor of ref len (default: 4)
    --dcv <int>          diff-cover period for blockwise (default: 1024)
    --nodc               disable diff-cover (algorithm becomes quadratic)
    -r/--noref           don't build .3/.4.ht2 (packed reference) portion
    -3/--justref         just build .3/.4.ht2 (packed reference) portion
    -o/--offrate <int>   SA is sampled every 2^offRate BWT chars (default: 5)
    -t/--ftabchars <int> # of chars consumed in initial lookup (default: 10)
    --localoffrate <int> SA (local) is sampled every 2^offRate BWT chars (default: 3)
    --localftabchars <int> # of chars consumed in initial lookup in a local index (default: 6)
    --snp <path>         SNP file name
    --haplotype <path>   haplotype file name
    --ss <path>          Splice site file name
    --exon <path>        Exon file name
    --seed <int>         seed for random number generator
    -q/--quiet           verbose output (for debugging)
    -h/--help           print detailed description of tool and its options
    --usage             print this usage message
    --version           print version information and quit
```

一部のモデル生物種以外は、リファレンス配列のインデックスを作る必要がある

## 実習1 hisat2-build

genome.faはArabidopsis thaliana (シロイヌナズナ)のレファレンスゲノム配列である。

中身を閲覧、query名およびreads数を確認せよ。

```
$ less genome.fa
$ grep '>' genome.fa
$ grep '>' genome.fa | wc
```

indexを作製せよ。

```
$ hisat2-build genome.fa genome
```

新たに作製されたファイルを確認せよ。

```
$ ls
```

# HISAT2基本コマンド

---

```
$ hisat2 -h
HISAT2 version 2.1.0 by Daehwan Kim (infphilo@gmail.com,
www.ccb.jhu.edu/people/infphilo)
Usage:
  hisat2 [options]* -x <ht2-idx> {-1 <m1> -2 <m2> | -U <r> | --sra-acc <SRA accession
number>} [-S <sam>]

<ht2-idx>  Index filename prefix (minus trailing .X.ht2).
<m1>       Files with #1 mates, paired with files in <m2>.
           Could be gzip'ed (extension: .gz) or bzip2'ed (extension: .bz2).
<m2>       Files with #2 mates, paired with files in <m1>.
           Could be gzip'ed (extension: .gz) or bzip2'ed (extension: .bz2).
<r>        Files with unpaired reads.
           Could be gzip'ed (extension: .gz) or bzip2'ed (extension: .bz2).
<SRA accession number>  Comma-separated list of SRA accession numbers, e.g. --
sra-acc SRR353653,SRR353654.
<sam>      File for SAM output (default: stdout)

<m1>, <m2>, <r> can be comma-separated lists (no whitespace) and can be
specified many times.  E.g. '-U file1.fq,file2.fq -U file3.fq'.
```

結果はsamファイルで出力される

## 実習2 hisat2

read結果

2D2L\_rep1\_R1.fastq

2D2L\_rep1\_R2.fastq

を先にindexを作製したリファレンスにmapさせよ。

```
$ hisat2 -p 4 --dta ¥  
-x genome ¥  
-1 2D2L_rep1_R1.fastq ¥  
-2 2D2L_rep1_R2.fastq ¥  
-S 2D2L_rep1.sam
```

samファイルの内容を確認しよう

```
$ less 2D2L_rep1.sam
```



# StringTie

Transcript assembly and quantification for RNA-Seq



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Manual

FAQ

CCB » Software » StringTie

- Overview
- News
- Obtaining and installing StringTie
- Licensing and contact Information
- Publications

## Overview

**StringTie** is a fast and highly efficient assembler of RNA-Seq alignments into potential transcripts. It uses a novel network flow algorithm as well as an optional *de novo* assembly step to assemble and quantitate full-length transcripts representing multiple splice variants for each gene locus. Its input can include not only alignments of short reads that can also be used by other transcript assemblers, but also alignments of longer sequences that have been assembled from those reads. In order to identify differentially expressed genes between experiments, StringTie's output can be processed by specialized software like [Ballgown](#), [Cuffdiff](#) or other programs (DESeq2, edgeR, etc.).

## News

▷ 3/9/2021 - v2.1.5 release changes:

- adjusted the calculation of coverage for long reads assembly
- new trimming procedure implemented

ダウンロードリンクはv2.1.6



# StringTieを用いてアラインされたreadを数える

---

StringTieの解析の方向性として大きく2つある

- ・GTFファイルに記載された遺伝子モデルのみを数える
- ・新規な遺伝子モデルを見出し、それも数える  
新規な遺伝子モデルはサンプルによって異なりうるので、  
個々のモデルをStringTieのmerge modeでmergeし、  
それを含めた、新しい遺伝子モデルを作製できる

# StringTie基本コマンド

---

```
$ stringtie
```

```
StringTie v2.1.6 usage:
```

```
stringtie <in.bam ..> [-G <guide_gff>] [-l <prefix>] [-o <out.gtf>] [-p <cpus>]
  [-v] [-a <min_anchor_len>] [-m <min_len>] [-j <min_anchor_cov>] [-f <min_iso>]
  [-c <min_bundle_cov>] [-g <bdist>] [-u] [-L] [-e] [--viral] [-E <err_margin>]
  [--ptf <f_tab>] [-x <seqid,..>] [-A <gene_abund.out>] [-h] {-B|-b <dir_path>}
  [--mix] [--conservative] [--rf] [--fr]
```

Assemble RNA-Seq alignments into potential transcripts.

Options:

```
--version : print just the version at stdout and exit
--conservative : conservative transcript assembly, same as -t -c 1.5 -f 0.05
--mix : both short and long read data alignments are provided
--rf : assume stranded library fr-firststrand
--fr : assume stranded library fr-secondstrand
-G reference annotation to use for guiding the assembly process (GTF/GFF3)
--ptf : load point-features from a given 4 column feature file <f_tab>
-o output path/file name for the assembled transcripts GTF (default: stdout)
:
:
:
```





- Running StringTie
- Input files
- Output files
- Evaluating transcript assemblies
- Differential expression analysis
  - Using StringTie with DESeq2 and edgeR
- Assembling super-reads

### Running StringTie

Run `stringtie` from the command line like this:

```
stringtie <aligned_reads.bam> [options]*
```

inputはsortされたBAM

The main input of the program is a BAM file with RNA-Seq read mappings which must be sorted by their genomic location (for example the `accepted_hits.bam` file produced by [TopHat](#) or the output of [HISAT2](#) after sorting and converting it using [samtools](#) as explained below).

The following optional parameters can be specified when running `stringtie`:

<code>-h/--help</code>	Prints help message and exits.
<code>-v</code>	Turns on verbose mode, printing bundle processing details.
<code>-o [&lt;path/&gt;]&lt;out.gtf&gt;</code>	Sets the name of the output GTF file where StringTie will write the assembled transcripts. This can be specified as a full path, in which case directories will be created as needed. By default StringTie writes the GTF at standard output.
<code>-p &lt;int&gt;</code>	Specify the number of processing threads (CPUs) to use for transcript assembly. The default is 1.
<code>-G &lt;ref_ann.gff&gt;</code>	Use the reference annotation file (in GTF or GFF3 format) to guide the assembly process. The output will include expressed reference transcripts as well as any novel transcripts that are assembled. This option is required by options <code>-B</code> , <code>-b</code> , <code>-e</code> , <code>-C</code> (see below).

```
$ stringtie ¥  
-e ¥  
-p 4 ¥  
-G genes.gtf ¥  
-o count_genes.gtf ¥  
hoge.sort.bam
```

- G reference annotation to use for guiding the assembly process (GTF/GFF3)
- e only estimate the abundance of given reference transcripts (requires -G)
- p number of threads (CPUs) to use (default: 1)
- o output path/file name for the assembled transcripts GTF (default: stdout)
- B enable output of Ballgown table files which will be created in the same directory as the output GTF (requires -G, -o recommended)

個々のサンプルごとに行う

## 実習3 stringtie

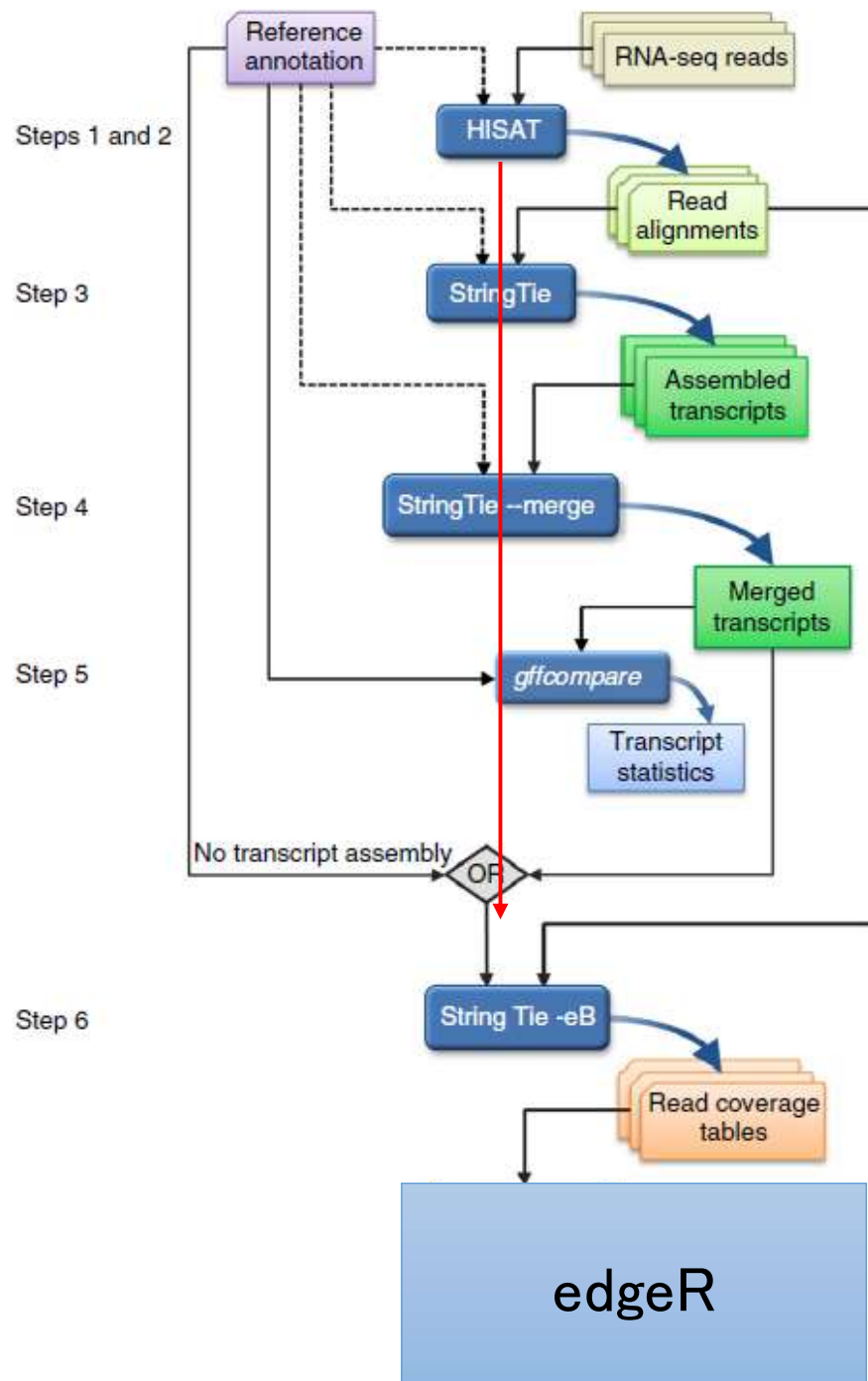
HISATで作製したsamをsort.bamにし、StringTieにかける

hisat結果 2D2L\_rep1.sam

```
$ samtools sort ¥  
-@ 4 ¥  
-o 2D2L_rep1.sort.bam ¥  
2D2L_rep1.sam  
  
$ stringtie -e -p 4 ¥  
-G genes.gtf ¥  
-o count_2D2L_rep1.gtf ¥  
2D2L_rep1.sort.bam
```

samtools v1.3以降、  
samファイルのsort,bam化  
は同時にできる





## Case B

新規な遺伝子モデルを見出し、  
それも数える場合

```
$ stringtie ¥  
-p 4 ¥  
-G genes.gtf ¥  
-o count_genes.gtf ¥  
hoge.sort.bam
```

- G reference annotation to use for guiding the assembly process (GTF/GFF3)
- p number of threads (CPUs) to use (default: 1)
- o output path/file name for the assembled transcripts GTF (default: stdout)
- e の指定はなし

個々のサンプルごとに行う

## StringTieのmerge modeでmerged\_gtfファイルを作製する

```
$ stringtie ¥  
--merge ¥  
-p 4 ¥  
-G genes.gtf ¥  
-o stringtie_merged.gtf ¥  
sample.list      #個々のgtfファイルのスペース区切りでの羅列も可
```

Sample.list

gtfファイルの場所を指定

Ex)

```
count_2D_rep1.gtf  
count_2D_rep2.gtf  
count_2D_rep3.gtf  
count_2D2L_rep1.gtf  
count_2D2L_rep2.gtf  
count_2D2L_rep3.gtf
```

mergeしたgtfファイルを-Gで指定して、先と同様-eを指定し、  
個々のbamからカウントデータを得る

```
$ stringtie ¥  
-e ¥  
-p 4 ¥  
-G stringtie_merged.gtf ¥  
-o count_genes.gtf ¥  
hoge.sort.bam
```

- G reference annotation to use for guiding the assembly process (GTF/GFF3)
- e only estimate the abundance of given reference transcripts (requires -G)
- p number of threads (CPUs) to use (default: 1)
- o output path/file name for the assembled transcripts GTF (default: stdout)

個々のサンプルでおこなう

# gtfファイルを比較するツール

## The gffcompare utility

The program `gffcompare` can be used to compare, merge, annotate and estimate accuracy of one or more GFF files (the "query" files), when compared with a reference annotation (also provided as GFF/GTF). A more detailed documentation for the program and its output files can be found [here](https://ccb.jhu.edu/software/stringtie/gff.shtml#gffcompare) ([gffcompare documentation page](https://ccb.jhu.edu/software/stringtie/gff.shtml#gffcompare))

<https://ccb.jhu.edu/software/stringtie/gff.shtml#gffcompare>

```
gffcompare ¥
-r gene.gtf ¥
-o merged ¥
stringtie_merged.gtf
```

gene.gtf <-既知model  
stringtie\_merged.gtf <-含新規model  
この両者を比較できる

```
# gffcompare v0.10.4 | Command line was:
#gffcompare -r genes.gtf -o merged stringtie_merged.gtf
#

#= Summary for dataset: stringtie_merged.gtf
#   Query mRNAs :   42241 in   33367 loci (30667 multi-exon
transcripts)
#               (6233 multi-transcript loci, ~1.3 transcripts per
locus)
# Reference mRNAs :   41607 in   33350 loci (30127 multi-exon)
# Super-loci w/ reference transcripts:   33240
#-----| Sensitivity | Precision |
Base level:   100.0    |   99.8    |
Exon level:   100.0    |   99.4    |
Intron level:  100.0    |   99.8    |
Intron chain level: 100.0 |   98.2    |
Transcript level: 100.0 |   98.5    |
Locus level:  100.0    |   99.9    |

Matching intron chains: 30127
Matching transcripts: 41607
Matching loci: 33350

Missed exons:      0/169264 ( 0.0%)
Novel exons:      102/170581 ( 0.1%)
Missed introns:    0/127896 ( 0.0%)
Novel introns:     55/128111 ( 0.0%)
Missed loci:       0/33350 ( 0.0%)
Novel loci:       37/33367 ( 0.1%)
```

# Differential expression analysis^

---

## Differential expression analysis

Together with [HISAT](#) and [Ballgown](#), StringTie can be used for estimating differential expression across multiple RNA-Seq samples and generating plots and differential expression tables as described in our [protocol paper](#).

⋮

## Using StringTie with DESeq2 and edgeR

DESeq2 and [edgeR](#) are two popular [Bioconductor](#) packages for analyzing differential expression, which take as input a matrix of read counts mapped to particular genomic features (e.g., genes). We provide a [Python script \(prepDE.py, or the Python 3 version: prepDE.py3\)](#) that can be used to extract this read count information directly from the files generated by StringTie (run with the `-e` parameter).



# カウントマトリックス作製

---

```
$ python prepDE.py -h
Usage: prepDE.py [options]
```

Generates two CSV files containing the count matrices for genes and transcripts, using the coverage values found in the output of `stringtie -e`

## Options:

```
-h, --help                show this help message and exit
-i INPUT, --input=INPUT, --in=INPUT
                           the parent directory of the sample sub-directories or
                           a textfile listing the paths to GTF files [default:
                           ballgown]
-g G                      where to output the gene count matrix [default:
                           gene_count_matrix.csv]
-t T                      where to output the transcript count matrix [default:
                           transcript_count_matrix.csv]
-l LENGTH, --length=LENGTH
                           the average read length [default: 75]
-p PATTERN, --pattern=PATTERN
                           a regular expression that selects the sample
                           subdirectories
-c, --cluster             whether to cluster genes that overlap with different
                           gene IDs, ignoring ones with geneID pattern (see
                           below)
-s STRING, --string=STRING
                           if a different prefix is used for geneIDs assigned by
                           StringTie [default: MSTRG]
-k KEY, --key=KEY         if clustering, what prefix to use for geneIDs assigned
                           by this script [default: prepG]
--legend=LEGEND           if clustering, where to output the legend file mapping
                           transcripts to assigned geneIDs [default: legend.csv]
```

```
$ python prepDE.py
```

```
gene_count_matrix.csv
```

```
transcript_count_matrix.csv
```

Case study 2: Genome-based RNA-Seq pipeline  
を進め、確認してみよう。

```
gene_id,2D2L_rep1,2D2L_rep2,2D2L_rep3,2D2L_rep4,2D_rep1,2D_rep2,2D_rep3,4D_rep1,4
D_rep2,4D_rep3,4D_rep4
AT4G22890,295,204,203,154,20,22,17,35,26,17,22
AT1G38440,0,0,0,0,0,0,0,0,0,0,0
AT3G27910,0,0,0,0,0,0,0,0,0,0,0
AT1G06620,3,0,6,0,0,3,4,9,0,3,0
AT5G54067,0,0,0,0,0,0,0,0,0,0,0
AT2G34630,52,13,10,18,9,0,3,11,7,12,11
AT2G46660,0,0,0,3,4,0,0,16,23,3,6
AT2G25590,13,7,7,12,3,4,7,21,15,13,15
AT1G43171,0,0,0,0,0,0,0,0,0,0,0
AT5G25130,3,5,3,5,0,0,0,0,0,0,0
AT2G32280,6,0,7,0,5,0,15,0,5,6,0
AT3G15020,5,0,4,7,40,9,23,9,18,10,0
AT5G61100,0,0,0,0,0,0,0,0,0,0,0
AT5G01650,42,15,27,13,35,19,33,0,23,10,18
AT5G05570,6,8,4,4,3,5,3,0,11,9,3
AT3G09770,47,30,25,10,3,14,14,38,46,13,26
AT3G10210,9,0,5,12,0,7,12,20,9,9,3
AT5G06000,0,0,0,5,7,0,5,0,0,0,0
AT5G64620,40,31,20,31,64,35,41,21,37,41,36
AT1G75280,36,45,36,44,8,11,14,16,10,4,11
```

このカウントマトリックスファイルをedgeRへのinputとして、transcript base解析で扱った同一の方法で解析を進める。

# edgeRでの解析

---

このケースでは ,が区切りのテキストとして得られているので、read.csvを用いる。

```
$ R
> library(edgeR)
> dat<-read.csv("gene_count_matrix.csv",row.names=1)
> group <- c(rep("2D",3),rep("2D2L",3))
> D<-DGEList(dat,group=group)
> D<-calcNormFactors(D)
> D<-estimateCommonDisp(D)
> D<-estimateTagwiseDisp(D)
```

2D vs 2D2Lの比較

```
> de_2D_2D2L <- exactTest(D,pair=c("2D","2D2L"))
> tmp <- topTags(de_2D_2D2L, n=nrow(de_2D_2D2L$table))
> write.table(tmp$table, "de.tagwise2.txt", sep="¥t", quote=F)
```

## まとめ

# HISAT

# StringTie

# edgeR

上記の流れを基盤にした、  
genome baseのDEG解析を紹介した

