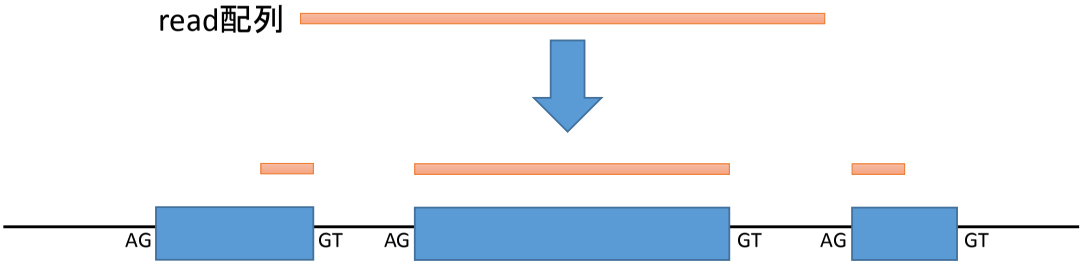
基生研ゲノムインフォマティックス・トレーニングコース　2017春
RNA-seq入門 – NGSの基礎からde novo解析まで
実践編 ： RNA-seq解析パイプライン
2017.03.09-2017.03.10

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

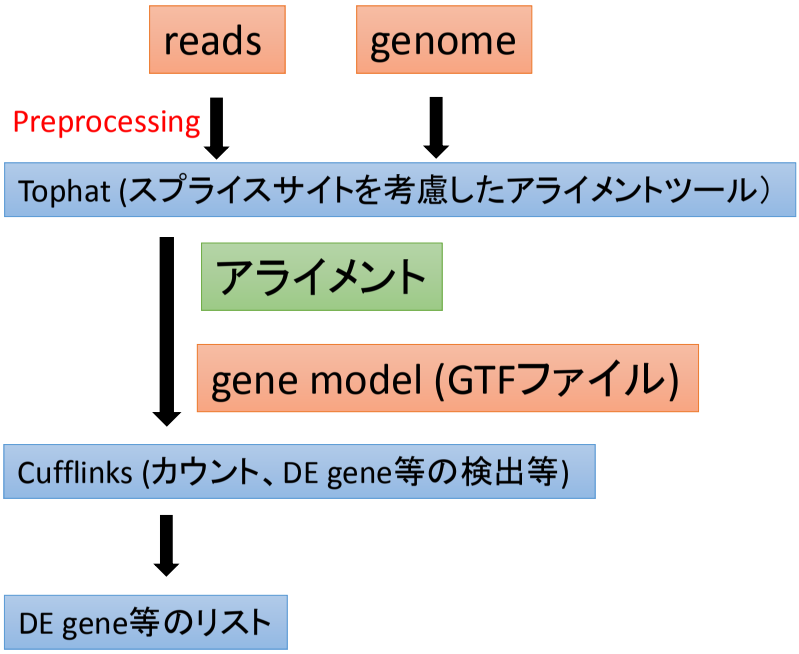
genomeをレファレンスとする場合

レファレンス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。
TopHatを用いる
他　Blat, SpliceMap, MapSplice, GSMAP, QPALMA

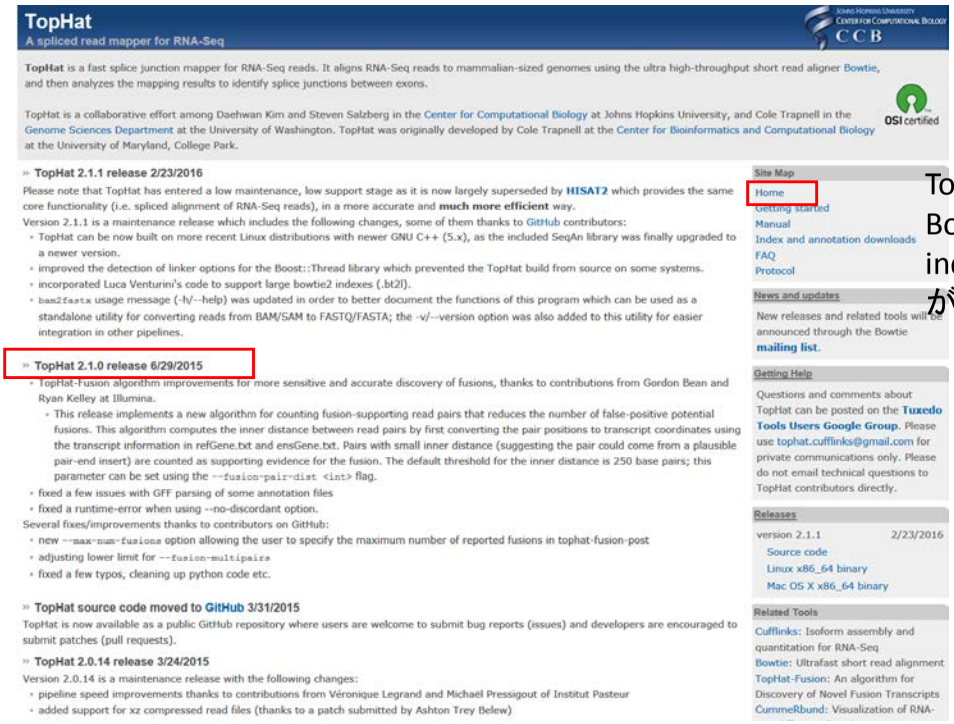


実際こんな感じにアラインされる

本トレーニングコースでの流れ



Tophat



TopHat
A spliced read mapper for RNA-Seq

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Please note that TopHat has entered a low maintenance, low support stage as it is now largely superseded by **HISAT2** which provides the same core functionality (i.e. spliced alignment of RNA-Seq reads), in a more accurate and **much more efficient** way.

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CummeRbund: Visualization of RNA-

TopHat2になりalignerとして
Bowtie2に対応
indelを考慮したアライメント
が可能になった　2012.4

Kim *et al. Genome Biology* 2013, **14**:R36
http://genomebiology.com/2013/14/4/R36

Genome Biology

METHOD Open Access

TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions

Daehwan Kim[1,2,3*], Geo Pertea[3], Cole Trapnell[5,6], Harold Pimentel[7], Ryan Kelley[8] and Steven L Salzberg[3,4]

Abstract

TopHat is a popular spliced aligner for RNA-sequence (RNA-seq) experiments. In this paper, we describe TopHat2, which incorporates many significant enhancements to TopHat. TopHat2 can align reads of various lengths produced by the latest sequencing technologies, while allowing for variable-length indels with respect to the reference genome. In addition to *de novo* spliced alignment, TopHat2 can align reads across fusion breaks, which can occur after genomic translocations. TopHat2 combines the ability to identify novel splice sites with direct mapping to known transcripts, producing sensitive and accurate alignments, even for highly repetitive genomes or in the presence of pseudogenes. TopHat2 is available at http://ccb.jhu.edu/software/tophat.

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A spliced read mapper for RNA-Seq

Johns Hopkins University Center for Computational Biology CCB

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OSI certified

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Getting startedで、とりあえず使ってみる

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version 2.1.1 2/23/2016

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Getting started

- Install quick-start
- Test the installation
- Preparing your reference
- Preparing your reads
- Running TopHat
- Examining your results

» **Install quick-start**

Download and extract the latest Bowtie 2 (or Bowtie) releases.

Note that you can use either Bowtie 2 (the default) or Bowtie (--bowtie1) and you will need the following Bowtie 2 (or Bowtie) programs in your PATH:

- `bowtie2` (or `bowtie`)
- `bowtie2-build` (or `bowtie-build`)
- `bowtie2-inspect` (or `bowtie-inspect`)

Installing a pre-compiled binary release

In order to make it easy to install TopHat we provide a few binary packages to save users from the occasionally frustrating process of building TopHat themselves, which requires a certain development environment and the Boost libraries installed. To use the binary packages, simply download the appropriate one for your platform, unpack it, and make sure the `tophat` binaries are in a directory in your PATH environment variable (or create a symbolic link to the included tophat2 script somewhere in your PATH, see below)

Note: if you want to be able to install and run this new version without overwriting a previous TopHat version already installed on your system, make sure you unpack the new version into a different directory from the old version, then instead of copying the new programs in a directory in your PATH just create a symbolic link from the `tophat2` wrapper script in this new directory to a directory in your shell's PATH. For example, assuming the ~/bin directory is in your PATH and you unpack tophat-2.0.0.Linux_x86_64.tar.gz under your home directory:

```
cd
tar xvfz tophat-2.0.0.Linux_x86_64.tar.gz
cd ~/bin
ln -s ~/tophat-2.0.0.Linux_x86_64/tophat2 .
```

Now you can start the new version of TopHat with the `tophat2` command, while the previous version, if present, can still be launched with the regular "tophat" command (assuming this is how you used it before).

Building TopHat from source

In order to build TopHat2 you must have the following installed on your system:

- the Boost C++ libraries (we recommend version 1.47 or higher so you can use it for building Cufflinks as well)

インストールの方法・
必要ツールなどの記載・
テストデータ等での極く簡単な
解析手順に関する記載がある

必要ツール
・bowtie2
・samtools

TopHat2はあらかじめコンパイルしたバイナリーファイルが配布されているので、自分でmakeする必要はない。
自分でソースからmakeする場合は
・SAMtools lib
・Boost C++ library
が必要

testデータが用意されている

```
tar zxvf test_data.tar.gz
cd test_data
tophat -r 20 test_ref reads_1.fq reads_2.fq
```

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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

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version 2.1.1 2/23/2016
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Manual

- What is TopHat?
- Prerequisites
- Using TopHat

・75base以上のreadに最適化
・リファレンス annotationなしでも
スプライスジャンクションを見つける

What is TopHat?

TopHat is a program that aligns RNA-Seq reads to a genome in order to identify exon-exon splice junctions. It is built on the ultrafast short read mapping program Bowtie. TopHat runs on **Linux** and **OS X**.

What types of reads can I use TopHat with?

TopHat was designed to work with reads produced by the Illumina Genome Analyzer, although users have been successful in using TopHat with reads from other technologies. In TopHat 1.1.0, we began supporting Applied Biosystems' Colorspace format. The software is optimized for reads 75bp or longer.

How does TopHat find junctions?

TopHat can find splice junctions without a reference annotation. By first mapping RNA-Seq reads to the genome, TopHat identifies potential exons, since many RNA-Seq reads will contiguously align to the genome. Using this initial mapping information, TopHat builds a database of possible splice junctions and then maps the reads against these junctions to confirm them.

Short read sequencing machines can currently produce reads 100bp or longer but many exons are shorter than this so they would be missed in the initial mapping. TopHat solves this problem mainly by splitting all input reads into smaller segments which are then mapped independently. The segment alignments are put back together in a final step of the program to produce the end-to-end read alignments.

TopHat generates its database of possible splice junctions from two sources of evidence. The first and strongest source of evidence for a splice junction is when two segments from the same read (for reads of at least 45bp) are mapped at a certain distance on the same genomic sequence or when an internal segment fails to map - again suggesting that such reads are spanning multiple exons. With this approach, "GT-AG", "GC-AG" and "AT-AC" introns will be found ab initio. The second source is pairings of "coverage islands", which are distinct regions of piled up reads in the initial mapping. Neighboring islands are often spliced together in the transcriptome, so TopHat looks for ways to join these with an intron. We only suggest users use this second option (--coverage-search) for short reads (< 45bp) and with a small number of reads (<= 10 million). This latter option will only report alignments across "GT-AG" introns

Illumina has provided the RNA-Seq user community with a set of genome sequence indexes (including Bowtie indexes) as well as GTF transcript annotation files. These files can be used with TopHat and Cufflinks to quickly perform expression analysis and gene discovery. The annotation files are augmented with the `tss_id` and `p_id` GTF attributes that Cufflinks needs to perform differential splicing, CDS output, and promoter user analysis. We recommend that you download your Bowtie indexes and annotation files from this page. More information about Illumina's iGenomes project can be found here.

Organism	Data source	Version	Size	Last Modified
Homo sapiens	Ensembl	GRCh37	17297 MB	May 14 17:23
	NCBI	build36.3	15814 MB	May 14 19:36
		build37.1	15850 MB	May 14 19:04
		build37.2	21450 MB	May 14 17:54
	UCSC	hg18	17349 MB	May 14 15:31
		hg19	21058 MB	May 14 15:36
Mus musculus	Ensembl	NCBIM37	14428 MB	May 14 22:13
	NCBI	build37.1	15260 MB	May 15 17:53
		build37.2	15725 MB	May 14 22:52
	UCSC	mm9	14537 MB	May 14 21:12
		mm10	14193 MB	Jun 14 11:29
Rattus norvegicus	Ensembl	RGSC3.4	13725 MB	May 15 22:33
	NCBI	RGSC_v3.4	14234 MB	May 15 23:58
	UCSC	rn4	13710 MB	May 15 22:32
Bos taurus	Ensembl	Btau_4.0	13315 MB	May 11 14:18
		UMD3.1	14042 MB	May 11 12:41
	NCBI	Btau_4.2	13357 MB	May 11 14:11
		Btau_4.6.1	13448 MB	May 11 16:09
		UMD_3.1	13990 MB	May 11 16:08

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TopHat-Fusion: An algorithm for

メジャーな生物種ではindexファイルやannotationファイル等が配布されているので有効活用できる

NATURE PROTOCOLS | PROTOCOL

Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks

Cole Trapnell, Adam Roberts, Loyal Goff, Geo Pertea, Daehwan Kim, David R Kelley, Harold Pimentel, Steven L Salzberg, John L Rinn & Lior Pachter

Affiliations | **Contributions** | **Corresponding author**

Nature Protocols **7**, 562–578 (2012) | doi:10.1038/nprot.2012.016
Published online 01 March 2012

protocol論文も出ている

ただし今となっては少し古い

Abstract

Abstract • Accession codes • References • Author information

Recent advances in high-throughput cDNA sequencing (RNA-seq) can reveal new genes and splice variants and quantify expression genome-wide in a single assay. The volume and complexity of data from RNA-seq experiments necessitate scalable, fast and mathematically principled analysis software. TopHat and Cufflinks are free, open-source software tools for gene discovery and comprehensive expression analysis of high-throughput mRNA sequencing (RNA-seq) data. Together, they allow biologists to identify new genes and new splice variants of known ones, as well as compare gene and transcript expression under two or more conditions. This protocol describes in detail how to use TopHat and Cufflinks to perform such analyses. It also covers several accessory tools and utilities that aid in managing data, including CummeRbund, a tool for visualizing RNA-seq analysis results. Although the procedure assumes basic informatics skills, these tools assume little to no background with RNA-seq analysis and are meant for novices and experts alike. The protocol begins with raw sequencing reads and produces a transcriptome assembly, lists of differentially expressed and regulated genes and transcripts, and publication-quality visualizations of analysis results. The protocol's execution time depends on the volume of transcriptome sequencing data and available computing resources but takes less than 1 d of computer time for typical experiments and ~1 h of hands-on time.

Freeではない

tophat基本コマンド

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```
> tophat -G gene.gtf -o out_dir genome read_1.fastq read_2.fastq
```

-G/--GTF <GTF/GFF3 file>
まずgtfに基づき、トランスクリプトにmapさせ、ゲノム位置として戻す。
mapしないリードはゲノムから探す

tophatの出力

prep_reads.info
align_summary.txt
deletions.bed
insertions.bed
junctions.bed
accepted_hits.bam
unmapped.bam

sam/bam フォーマットのファイル
accepted_hits.bamファイルがこの後必要

実習1
tophatを用いてpreprocessing済みの2D_rep1.fastqファイルをgenome_chr4にmapさせよ
GTFファイルとしてgenes_chr4.gtfを用いる

例）これにファイルディレクトリーを加える

```
$ tophat -p 4 -G genes_chr4.gtf -o 2D_rep1 genome_chr4 2D_rep1_R1.fastq 2D_rep1_R2.fastq
```

出力を確認しよう。
例えば、align_summary.txtを見ればどの程度mapしたか分かる。
これでRNA-Seqのリード配列がゲノム配列にアラインできた。

cufflinksを用いてアラインされたreadを数える

定義した方法でのカウントが可能
gene単位
トランスクリプト単位
エキソン単位

- cufflinks
-BEDTools
-HTseq
が利用できる

今回はCufflinksを利用
そもそもTopHat → Cufflinksの解析系は同じ開発元、非常に良く使われている。

ローカスアノテーション情報を記載したgtfファイルを用意しておけば、
それに基づいて、genes単位、isoforms単位での解析を進めてくれる。

簡易的に、特定ローカスの解析などを進めたい場合や、
gtfファイルがない場合などは、BEDToolsも有用
gtfファイルを自分で作製するのは結構大変だが、bedファイルは比較的容易

http://cole-trapnell-lab.github.io/cufflinks/

Cufflinks

Transcriptome assembly and differential expression analysis for RNA-Seq.

Cufflinks assembles transcripts, estimates their abundances, and tests for differential expression and regulation in RNA-Seq samples. It accepts aligned RNA-Seq reads and assembles the alignments into a parsimonious set of transcripts. Cufflinks then estimates the relative abundances of these transcripts based on how many reads support each one, taking into account biases in library preparation protocols.

Cufflinks was originally developed as part of a collaborative effort between the Laboratory for Mathematical and Computational Biology, led by Lior Pachter at UC Berkeley, Steven Salzberg's computational genomics group at the Institute of Genetic Medicine at Johns Hopkins University, and Barbara Wold's lab at Caltech. The project is now maintained by Cole Trapnell's lab at the University of Washington.

Cufflinks is provided under the OSI-approved Boost License

News

*To get the latest updates on the Cufflinks project and the rest of the "Tuxedo tools", please subscribe to our **mailing list***

Cufflinks has moved to GitHub	DECEMBER 10, 2014
Cufflinks 2.2.1 released	MAY 05, 2014
Cufflinks 2.2.0 released	MARCH 25, 2014
Cufflinks 2.1.1 released	APRIL 11, 2013

Protocol

Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation

Cole Trapnell, Brian A Williams, Geo Pertea, Ali Mortazavi, Gordon Kwan, Marijke J van Baren, Steven L Salzberg, Barbara J Wold & Lior Pachter

Affiliations | Contributions | Corresponding author

Nature Biotechnology **28**, 511–515 (2010) | doi:10.1038/nbt.1621
Received 02 February 2010 | Accepted 22 March 2010 | Published online 02 May 2010

High-throughput mRNA sequencing (RNA-Seq) promises simultaneous transcript discovery and abundance estimation[1, 2, 3]. However, this would require algorithms that are not restricted by prior gene annotations and that account for alternative transcription and splicing. Here we introduce such algorithms in an open-source software program called Cufflinks. To test Cufflinks, we sequenced and analyzed >430 million paired 75-bp RNA-Seq reads from a mouse myoblast cell line over a differentiation time series. We detected 13,692 known transcripts and 3,724 previously unannotated ones, 62% of which are supported by independent expression data or by homologous genes in other species. Over the time series, 330 genes showed complete switches in the dominant transcription start site (TSS) or splice isoform, and we observed more subtle shifts in 1,304 other genes. These results suggest that Cufflinks can illuminate the substantial regulatory flexibility and complexity in even this well-studied model of muscle development and that it can improve transcriptome-based genome annotation.

INSTALL MANUAL GETTING STARTED TOOLS HELP HOW IT WORKS PROTOCOL BENCHMARKS CODE FEED

Cufflinks

Transcriptome assembly and differential expression analysis for RNA-Seq.

Cufflinks is available for Linux and Mac OS X. You can find the full list of releases below.

The Cufflinks source code for each point release is available below as well. If you want to grab the current code, check out the Cufflinks GitHub repository.

Star 68 Fork 48

Cufflinks Releases

Version	Date			
2.2.1	May 05, 2014	Linux	Mac OS X	Source
2.2.0	March 25, 2014	Linux	Mac OS X	Source
2.1.1	April 11, 2013	Linux	Mac OS X	Source
2.1.0	April 10, 2013	Linux	Mac OS X	Source

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Cufflinks

Transcriptome assembly and differential expression analysis for RNA-Seq.

自分でソースからmakeする場合は
・Samtools
・Boost C++ library
が必要

```
cufflinks ./test_data.sam
```

これでツールが動くことを確認

Install quick-start

Installing a pre-compiled binary release

In order to make it easy to install Cufflinks, we provide a few binary packages to save users from occasionally frustrating process of building Cufflinks, which requires that you install the Boost libraries. To use the binary packages, simply download the appropriate one for your machine, untar it, and make sure the cufflinks,cuffdiff and cuffcompare binaries are in a directory in your PATH environment variable.

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Cufflinks

Transcriptome assembly and differential expression analysis for RNA-Seq.

Bowtie: ultrafast short read alignment

Bowtie is an ultrafast and memory-efficient tool for aligning sequencing reads to long reference sequences. It is particularly good at aligning reads of about 50 up to 100s or 1,000s of characters, and particularly good at aligning to relatively long (e.g. mammalian) genomes. Bowtie 2 indexes the genome with an FM Index to keep its memory footprint small: for the human genome, its memory footprint is typically around 3.2 GB. Bowtie 2 supports gapped, local, and paired-end alignment modes.

Bowtie is provided under the OSI-approved Artistic License 2.0.

TopHat: alignment of short RNA-Seq reads

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 provided under the OSI-approved Artistic License 2.0.

CummeRbund: visualization of RNA-Seq differential analysis

CummeRbund is an R package that is designed to aid and simplify the task of analyzing Cufflinks RNA-Seq output.

CummeRbund is provided under the OSI-approved Artistic License 2.0.

Monocle: Differential expression for single-cell RNA-Seq and qPCR.

Monocle is a toolkit for analyzing single-cell gene expression experiments. Monocle was designed for RNA-Seq, but can also work with single cell qPCR. It performs differential expression analysis, and can find genes that differ between cell types or between cell states. When used to study an ongoing biological process such as cell differentiation, Monocle learns that process and places cells in order according to their progress through it. Monocle finds genes that are dynamically regulated during that process.

Monocle is provided under the OSI-approved Artistic License (version 2.0)

Cufflinksの関連ツール
Bowtie, TopHatは説明済み

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Cufflinks

Transcriptome assembly and differential expression analysis for RNA-Seq.

The Cufflinks RNA-Seq workflow

The Cufflinks suite of tools can be used to perform a number of different types of analyses for RNA-Seq experiments. The Cufflinks suite includes a number of different programs that work together to perform these analyses. The complete workflow, performing all the types of analyses Cufflinks can execute, is summarized in the graph below. The left side illustrates the "classic" RNA-Seq workflow, which includes read mapping with TopHat, assembly with Cufflinks, and visualization and exploration of results with CummeRbund. A newer, more advanced worfklow was introduce with Cufflinks version 2.2.0, and is shown on the right. Both are still supported. You can read about the classic workflow in detail in our protocol paper.

Cufflinks

Cufflinks is both the name of a suite of tools and a program within that suite. Cufflinks the program assembles transcriptomes from RNA-Seq data and quantifies their expression.

Cuffcompare

After assembling a transcriptome from one or more samples, you'll probably want to compare your assembly to known transcripts. Even if there is no "reference" transcriptome for the organism you're studying, you may want to compare the transcriptomes assembled from different RNA-Seq libraries. Cuffcompare helps you perform these comparisons and assess the quality of your assembly.

Cuffmerge

When you have multiple RNA-Seq libraries and you've assembled transcriptomes from each of them, we recommend that you merge these assemblies into a master transcriptome. This step is required for a differential expression analysis of the new transcripts you've assembled. Cuffmerge performs this merge step.

Cuffquant

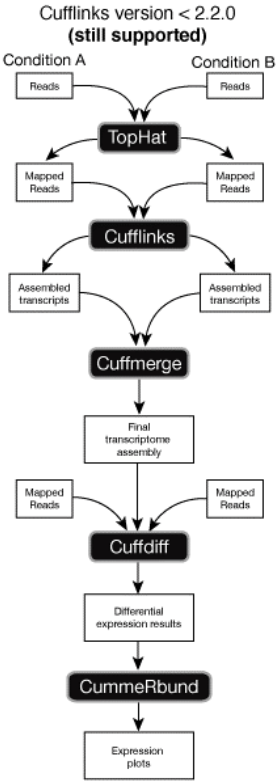
Quantifying gene and transcript expression in RNA-Seq samples can be computationally expensive. Cuffquant allows you to compute the gene and transcript expression profiles and save these profiles to files that you can analyze later with Cuffdiff or Cuffnorm. This can help you distribute your computational load over a cluster and is recommended for analyses involving more than a handful of libraries.

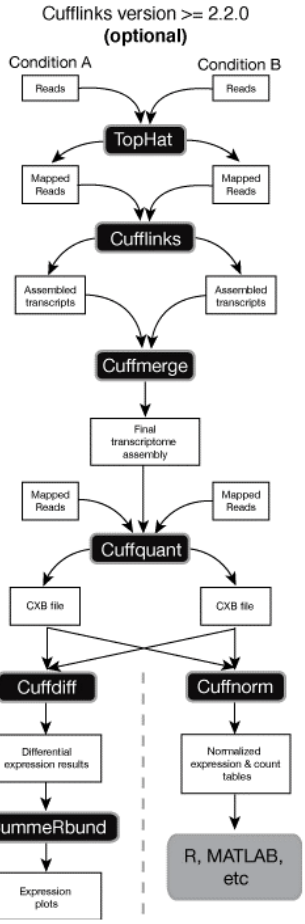
Cuffdiff

Comparing expression levels of genes and transcripts in RNA-Seq experiments is a hard problem. Cuffdiff is a highly accurate tool for performing these comparisons, and can tell you not only which genes are up- or down-regulated between two or more conditions, but also which genes are differentially spliced or are undergoing other types of isoform-level regulation.

Cuffnorm

Sometimes, all you want to do is normalize the expression levels from a set of RNA-Seq libraries so that they're all on the same scale, facilitating downstream analyses such as clustering. Expression levels reported by Cufflinks in FPKM units are usually comparable between samples, but in certain situations, applying an extra level of normalization can remove sources of bias in the data. Cuffnorm normalizes a set of samples to be on as similar scales as possible, which can improve the results you obtain with other downstream tools.





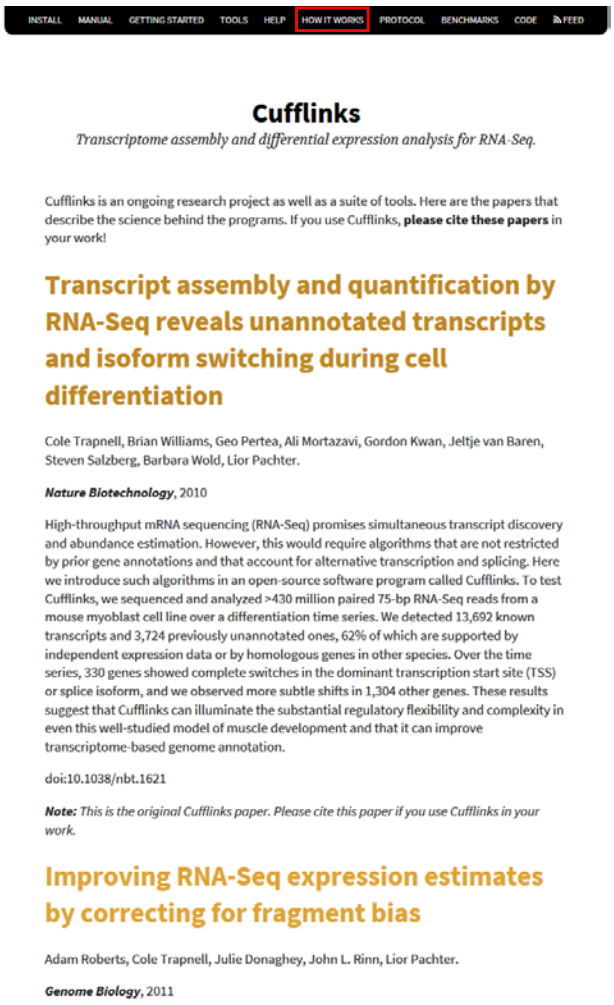
cufflink

cufflinks
cuffmerge
cuffcompare
cuffquant
cuffnorm
cuffdiff
の6つのプログラムから構成

cuffquant, cuffnormは
ver2.2.0(20140325)
から実装

MacOSX版のバイナリーはver2.2.0以降はバグがありsegmentation errorでまともに動かないようです。

今回の実習ではver2.1.1を使用し、cuffquant, cuffnormは簡単な説明のみに留めます。



どうやって動いているか

まず動いて使えそうな感じに
なったら詳細を把握していく

cufflinks基本コマンド

Cufflinksコマンド

cufflinks -o out_directory -G hoge.gtf tophat_directory/accepted_hits.bam

cufflinksを実行してパラメータを確認しよう。

考慮すべきパラメーター例

-o	出力の指定、TopHatの出力と同じ場所にしておくのが分かりやすいだろう
-p	CPUスレッド数の指定(デフォルトは1)、結構時間がかかるので使える数を指定すると良いだろう
-G	GTFファイルに記載されたアノテーションのみについて計算
-g	GTFファイルに記載されたアノテーションをガイドにしてアセンブルする
-M	無視したいトランスクリプト（rRNAなど）を指定

cufflinks出力

出力
skipped.gtf
transcripts.gtf
genes.fpkm_tracking
isoforms.fpkm_trancking

実習2
先のtophatの結果を用いてcufflinksにかけてみよう

例）これにファイルディレクトリーを加える
```
> cufflinks -p 4 -o 2D_rep1 -G genes_chr4.gtf accepted_hits.bam
```

出力を確認しよう。
geneごと、isoformごとにFPKM値が計算されているのが分かる。
また-gを用いてcufflinksにかけると新規の発現領域が存在するのが分かる

cuffcompareコマンド

Cufflinks includes a program that you can use to help analyze the transfrags you assemble. The program cuffcompare helps you:
Compare your assembled transcripts to a reference annotation
Track Cufflinks transcripts across multiple experiments (e.g. across a time course)
From the command line, run cuffcompare as follows:

cuffcompare [options]* <cuff1.gtf> [cuff2.gtf] ... [cuffN.gtf]

今回はすでにあるgtfファイルの情報を用いるので、意識的に使う必要はない。

cuffmergeコマンドと出力

個々のサンプルのアセンブルモデルを統合する。

```
Usage:
    cuffmerge [Options] <assembly_GTF_list.txt>

Options:
    -h/--help                           Prints the help message and exits
    -o                  <output_dir>    Directory where merged assembly will be written  [ default: ./merged_asm  ]
    -g/--ref-gtf                        An optional "reference" annotation GTF.
    -s/--ref-sequence   <seq_dir>/<seq_fasta> Genomic DNA sequences for the reference.
    --min-isoform-fraction <0-1.0>      Discard isoforms with abundance below this       [ default:           0.05 ]
    -p/--num-threads    <int>           Use this many threads to merge assemblies.       [ default:              1  ]
    --keep-tmp
```

統合ファイルリストを事前に作製する必要がある(例 assemblies,txt)

cuffmerge -s $REFSEQ -g $GTF assemblies.txt

例 assemblies,txt

~/arabi_2D_2/transcripts.gtf
~/arabi_2D_3/transcripts.gtf
~/arabi_2D2L_2/transcripts.gtf
~/arabi_2D2L_3/transcripts.gtf

出力

merged.gtf

Cufflinks includes a script called cuffmerge that you can use to merge together several Cufflinks assemblies. It handles also handles running Cuffcompare for you, and automatically filters a number of transfrags that are probably artfifacts. If you have a reference GTF file available, you can provide it to the script in order to gracefully merge novel isoforms and known isoforms and maximize overall assembly quality. The main purpose of this script is to make it easier to make an assembly GTF file suitable for use with Cuffdiff.

今回はすでにあるgtfファイルの情報を用いるので、使う必要はない。

cuffdiffコマンド

DE gene等を統計計算で取り出す
コマンド入力して使用法を確認してみよう

```
Usage:   cuffdiff [options] <transcripts.gtf> <sample1_hits.sam> <sample2_hits.sam> [... sampleN_hits.sam]
Supply replicate SAMs as comma separated lists for each condition:
sample1_rep1.sam,sample1_rep2.sam,...sample1_repM.sam
General Options:
  -o/--output-dir             write all output files to this directory             [ default:     ./ ]
  -L/--labels                 comma-separated list of condition labels
  --FDR                       False discovery rate used in testing                 [ default:   0.05 ]
```

cuffdiff -o out_file merged.gtf bam1,bam2,bam3 bam4,bam5,bam6

Version 2.2.0以降は後述のcuffquantで得られたcxbファイルをbamファイルの代わりに用いる。
cuffdiffにかかる時間やメモリー使用量が軽減される。

cuffdiffの出力

bias_params.info
run.info
read_groups.info
var_model.info
cds.read_group_tracking
cds.fpkm_tracking
cds.count_tracking
genes.read_group_tracking
genes.fpkm_tracking
genes.count_tracking
isoforms.read_group_tracking
isoforms.count_tracking
isoforms.fpkm_tracking
tss_groups.read_group_tracking
tss_groups.fpkm_tracking
tss_groups.count_tracking

gene_exp.diff
cds_exp.diff
cds.diff
isoform_exp.diff
promoters.diff
splicing.diff
tss_group_exp.diff

diffの付いたファイルがそれぞれの
違いの情報を記載したファイル

.diffファイルの内容

Column number	Column name	Example	Description
1	Tested id	`XLOC_000001`	A unique identifier describing the transcipt, gene, primary transcript, or CDS being tested
2	gene	`Lypla1`	The `gene_name`(s) or `gene_id`(s) being tested
3	locus	`chr1:4797771-4835363`	Genomic coordinates for easy browsing to the genes or transcripts being tested.
4	sample 1	`Liver`	Label (or number if no labels provided) of the first sample being tested
5	sample 2	`Brain`	Label (or number if no labels provided) of the second sample being tested
6	Test status	`NOTEST`	Can be one of OK (test successful), NOTEST (not enough alignments for testing), LOWDATA (too complex or shallowly sequenced), HIDATA (too many fragments in locus), or FAIL, when an ill-conditioned covariance matrix or other numerical exception prevents testing.
7	$FPKM_x$	`8.01089`	FPKM of the gene in sample *x*
8	$FPKM_y$	`8.551545`	FPKM of the gene in sample *y*
9	log2 ($FPKM_y/FPKM_x$)	`0.06531`	The (base 2) log of the fold change *y*/*x*
10	test stat	`0.860902`	The value of the test statistic used to compute significance of the observed change in FPKM
11	p value	`0.389292`	The **uncorrected** *p*-value of the test statistic
12	q value	`0.985216`	The **FDR-adjusted** *p*-value of the test statistic
13	significant	`no`	Can be either "yes" or "no", depending on whether *p* is greater then the FDR **after** Benjamini-Hochberg correction for multiple-testing

cuffquantコマンドと出力(ver2.2.0以降)

bamの内容からgene/transcriptレベルで定量化し、バイナリー出力する

cuffquant -o out_directory hoge.gtf accepted_hits.bam

cuffquantを実行してパラメータを確認しよう。

考慮すべきパラメーター例
-o　　出力ディレクトリーの指定
-p　　CPUスレッド数の指定(デフォルトは1)、結構時間がかかるので使える数を指定
-M　　無視したいトランスクリプト（rRNAなど）を指定
他にもestimationに関わる　-b　-u パラメータがある。

出力
abundances.cxb

```
> cuffquant -p 4 -o 2D_1 genes_chr4.gtf accepted_hits.bam
```

新たにcxbファイルが作製されていることが分かる。
出力ファイルはこの１つだけ

cuffdiffの前にcuffquantを行い、cxbファイルを作製することで
cuffdiffを速くできる。

cuffnormコマンドと出力(ver2.2.0以降)

Cuffnormコマンド

Cuffnorm, which simply computes
a normalized table of expression values for genes and transcripts.

```
> cuffnorm -o out_file genes_chr4.gtf bam1,bam2,bam3 bam4,bam5,bam6
```

cuffnorm [options]* <transcripts.gtf>
<sample1_replicate1.sam[,...,sample1_replicateM.sam]>
<sample2_replicate1.sam[,...,sample2_replicateM.sam]>...
[sampleN.sam_replicate1.sam[,...,sample2_replicateM.sam]]

sam/bamかcxbファイルどちらも入力可能。ただし混在は不可

cuffnormの出力(ver2.2.0以降)

cds.attr_table
cds.count_table
cds.fpkm_table
cuffnorm.tree
genes.attr_table
genes.count_table
genes.fpkm_table
isoforms.attr_table
isoforms.count_table
isoforms.fpkm_table
run.info
samples.table
tss_groups.attr_table
tss_groups.count_table
tss_groups.fpkm_table

たくさんのサンプルで発現プロットやクラスター図を書きたい場合便利。

tophat -> cufflinksの解析系を使用する際の注意

It does not perform differential expression analysis. To assess the significance of changes in expression for genes and transcripts between conditions, use Cuffdiff. Cuffnorm's output files are useful when you have many samples and you simply want to cluster them or plot expression levels of genes important in your study.

Cuffnorm will report both FPKM values and **normalized**, estimates for the number of fragments that originate from each gene, transcript, TSS group, and CDS group. Note that because these counts are already normalized to account for differences in library size, they should not be used with downstream differential expression tools that require **raw** counts as input.

tophat -> cufflinksは一連の解析系

cufflinksの出力はすでにノーマライズされたものなので、rawデータを要求するedgeRなどの別のツールのinputには利用できない。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NATURE PROTOCOLS | PROTOCOL

Count-based differential expression analysis of RNA sequencing data using R and Bioconductor

Simon Anders, Davis J McCarthy, Yunshun Chen, Michal Okoniewski, Gordon K Smyth, Wolfgang Huber & Mark D Robinson

Affiliations | Contributions | Corresponding authors

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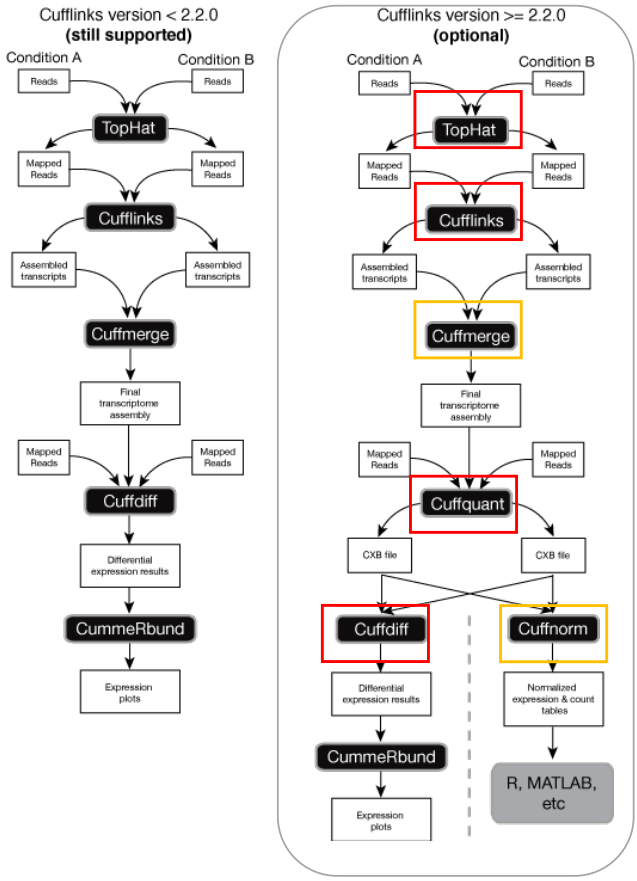
Abstract

Abstract • Accession codes • References • Author information • Supplementary information

RNA sequencing (RNA-seq) has been rapidly adopted for the profiling of transcriptomes in many areas of biology, including studies into gene regulation, development and disease. Of particular interest is the discovery of differentially expressed genes across different conditions (e.g., tissues, perturbations) while optionally adjusting for other systematic factors that affect the data-collection process. There are a number of subtle yet crucial aspects of these analyses, such as read counting, appropriate treatment of biological variability, quality control checks and appropriate setup of statistical modeling. Several variations have been presented in the literature, and there is a need for guidance on current best practices. This protocol presents a state-of-the-art computational and statistical RNA-seq differential expression analysis workflow largely based on the free open-source R language and Bioconductor software and, in particular, on two widely used tools, DESeq and edgeR. Hands-on time for typical small experiments (e.g., 4–10 samples) can be <1 h, with computation time <1 d using a standard desktop PC.

versionによる違いまとめ



tophat, cufflinksの実習

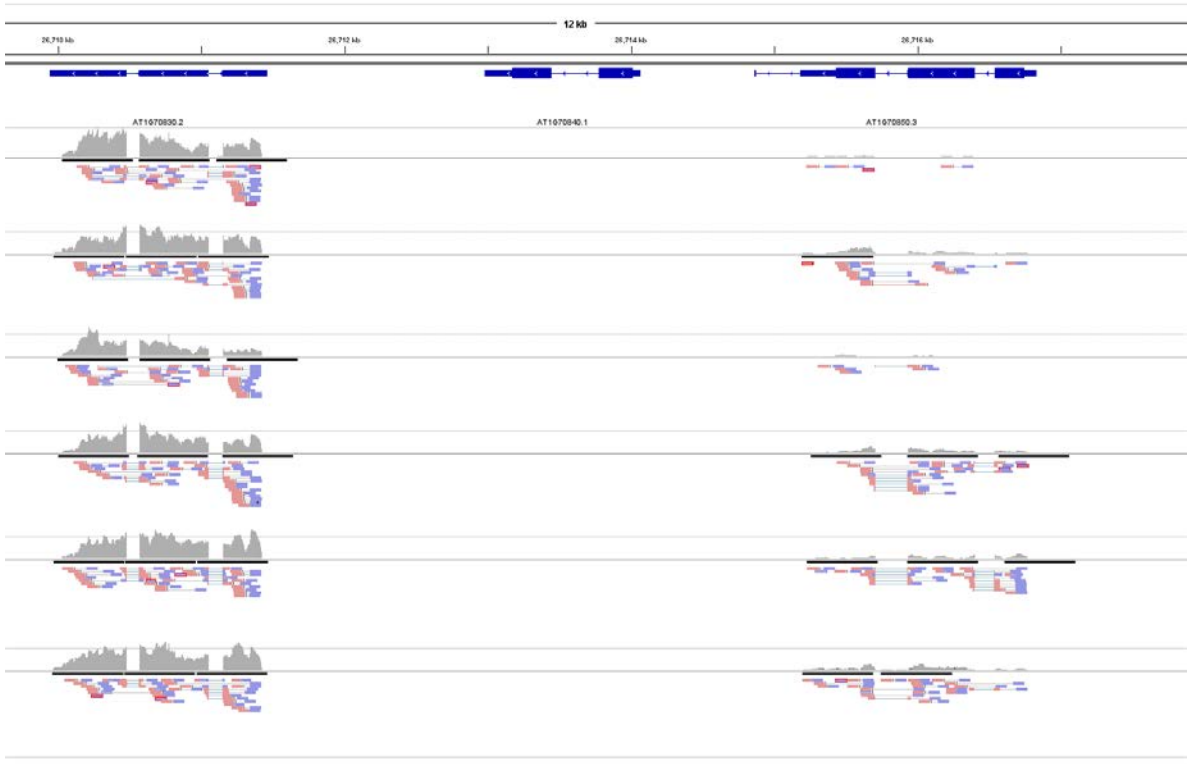
/data/KY/tophatにあるファイルを用いる。

1. TopHatを用いて、paired-endのtest data（2D_rep1_R1.fastq,2D_rep1_R2.fastq
をリファレンスgenome_chr4にマップさせよ
オプション -Gの有無に
よる違いを確認しよう。

2.Cufflinksを用いて、
2D_rep1のカウントをしよう。
-Gと-gの違いを確認しよう。

RNA-Seq結果のIGV実習

map結果をIGVで可視化してみよう
TAIR10の配列を呼び出し、TopHatで得られたBAMファイルをindexファイルを付け、読み込む



Excelを使って結果を確認してみよう

2D vs 2D2Lのcuffdiff結果が~data/KY/cuffdiffフォルダーにある。
Excelでgene_exp.diffファイルを読み込んでみる
tab区切りテキストファイルなのでそのまま読み込める
Excelのsort機能を使ってq値でsortしてみる

q値でsort

test_id	gene_id	gene	locus	sample_1	sample_2	status	value_1	value_2	log2(fold_chan	test_stat	p_value	q_value	significant
XLOC_000047	XLOC_000047	KEA1	1:284609-291094	q1	q2	OK	12.8356	47.6879	1.89347	4.44122	5.00E-05	0.000325	yes
XLOC_000091	XLOC_000091	BXL2	1:564204-567769	q1	q2	OK	112.839	21.5634	-2.38762	-6.02938	5.00E-05	0.000325	yes
XLOC_000148	XLOC_000148	PSB27	1:898875-899655	q1	q2	OK	194.744	691.64	1.82844	7.10401	5.00E-05	0.000325	yes
XLOC_000310	XLOC_000310	PSBP-1	1:2047824-2049418	q1	q2	OK	588.195	3147.84	2.42	7.92975	5.00E-05	0.000325	yes
XLOC_000404	XLOC_000404	NPQ1	1:2706923-2709531	q1	q2	OK	21.2494	78.5734	1.88662	3.26377	5.00E-05	0.000325	yes
XLOC_000419	XLOC_000419	CSD1	1:2827060-2838469	q1	q2	OK	503.523	181.545	-1.47173	-5.38312	5.00E-05	0.000325	yes
XLOC_000450	XLOC_000450	CSP41B	1:3015327-3018234	q1	q2	OK	113.687	650.406	2.51627	8.83387	5.00E-05	0.000325	yes
XLOC_000487	XLOC_000487	LRR XI-23	1:3252239-3255693	q1	q2	OK	26.4081	49.6396	0.910512	2.30664	5.00E-05	0.000325	yes
XLOC_000598	XLOC_000598	ATGLX1	1:3995168-3997907	q1	q2	OK	60.1583	162.387	1.4326	3.26419	5.00E-05	0.000325	yes
XLOC_000600	XLOC_000600	AT1G11860	1:4001112-4003442	q1	q2	OK	319.6	756.582	1.24323	4.18318	5.00E-05	0.000325	yes
XLOC_000614	XLOC_000614	AT1G12080	1:4084161-4085045	q1	q2	OK	1884.29	67.9613	-4.79316	-9.20293	5.00E-05	0.000325	yes
XLOC_000616	XLOC_000616	CHL1-1	1:4105232-4109545	q1	q2	OK	107.267	57.7917	-0.892267	-2.70294	5.00E-05	0.000325	yes
XLOC_000624	XLOC_000624	AT1G12230	1:4147961-4151056	q1	q2	OK	102.049	50.9296	-1.00268	-2.40566	5.00E-05	0.000325	yes
XLOC_000680	XLOC_000680	CYP71B7	1:4467219-4469033	q1	q2	OK	17.1443	84.588	2.30272	4.53043	5.00E-05	0.000325	yes
XLOC_000724	XLOC_000724	AT1G13930	1:4761011-4762666	q1	q2	OK	94.6747	2483.48	4.71324	10.4968	5.00E-05	0.000325	yes
XLOC_000749	XLOC_000749	AT1G14345	1:4899144-4899979	q1	q2	OK	38.3992	157.145	2.03295	4.49341	5.00E-05	0.000325	yes
XLOC_000765	XLOC_000765	AT1G14670	1:5037611-5040528	q1	q2	OK	84.8105	44.439	-0.932415	-2.66978	5.00E-05	0.000325	yes
XLOC_000835	XLOC_000835	NDF1	1:5489297-5493772	q1	q2	OK	20.0548	104.567	2.3824	4.27443	5.00E-05	0.000325	yes
XLOC_000884	XLOC_000884	HCF173	1:5723087-5727312	q1	q2	OK	7.34039	112.227	3.93442	5.2414	5.00E-05	0.000325	yes
XLOC_000916	XLOC_000916	FUG1	1:5885082-5890470	q1	q2	OK	48.9638	105.457	1.10687	3.5512	5.00E-05	0.000325	yes
XLOC_001003	XLOC_001003	NDF6	1:6460597-6462224	q1	q2	OK	45.3045	185.555	2.03412	2.97075	5.00E-05	0.000325	yes
XLOC_001030	XLOC_001030	LHCA6	1:6612748-6613972	q1	q2	OK	52.6816	153.395	1.54188	4.09397	5.00E-05	0.000325	yes
XLOC_001063	XLOC_001063	PUP14	1:6832346-6833837	q1	q2	OK	37.731	91.5568	1.27892	3.13218	5.00E-05	0.000325	yes
XLOC_001076	XLOC_001076	ATLFNR2	1:6942716-6945018	q1	q2	OK	87.7487	1025.37	3.54662	10.0816	5.00E-05	0.000325	yes
XLOC_001099	XLOC_001099	AT1G20390	1:7065493-7071561	q1	q2	OK	45.6232	15.9769	-1.51378	-4.22277	5.00E-05	0.000325	yes
XLOC_001170	XLOC_001170	AT1G21680	1:7613004-7615339	q1	q2	OK	27.146	80.96	1.57647	3.93831	5.00E-05	0.000325	yes

GTFファイルに記載された遺伝子ごとの発現カウントに対して倍率、p値、q値が計算される。

RをつかってMA plotを書いて見よう

先と同じgene_exp.diffファイルを読み込んでみる
tab区切りテキストファイルなのでread.delim関数で読み込む
M, Aをそれぞれ計算する
plot関数を使って描画
colorのパラメータをsignifitureの値で色分けさせてみる。

例)

```
dat <- read.delim("gene_exp.diff")
A<-1/2*(log2(dat$value_1+1)+log2(dat$value_2+1))
M<-log2(dat$value_1+1)-log2(dat$value_2+1)
plot(A,M,col=dat$significant, pch=16, cex=0.4, ylim=c(-8,8))
```

簡易スクリプトを使って、結果を成形してみよう

Awkによる1行スクリプトで、q_value値が0.05以下となる行を取り出せ。
またその数を数えよ。

例）
q_valueが0.05以下のもののみリストアップするには？
q_valueの記載は13列目だから・・・

```
awk '$13<=0.05 {print $0}' gene_exp.diff
```

と記述すればOK
$で列番号を指定できる
$0は行全体を意味する

```
awk '$13<=0.05 {print $0}' gene_exp.diff | wc
```

で数も分かる。

実践演習課題

cutadapt済みのデータセット~data/KY/tophatフォルダーの
2D_rep*_R*.fastq（2days dark条件で育てたアラビドプシス芽生え),
2D2L_rep*_R*.fastq（その後2days light条件で育てたアラビドプシス芽生え）
それぞれ3反復のデータ　を用い TopHat→Cufflinksの系を用い、
DE gene等を調べよ。

GTFファイルとしてgenes_chr4.gtf
fastaファイルとしてgenome_chr4.fa
を利用する。
（アラビドプシスTAIR10の配列だが計算時間を考慮して、
それぞれChr4のみになっている）

RNA-Seqパイプライン -ゲノムベースの解析法-の最後3スライドを参考に、
マッピングデータのIGVでの可視化、
エクセルでの確認、
Rを用いたM-A plotの描画、
例に挙げた流れで、簡易スクリプトを用いたデータ抽出をせよ。