RNA-seq解析パイプライン：Transcript-based

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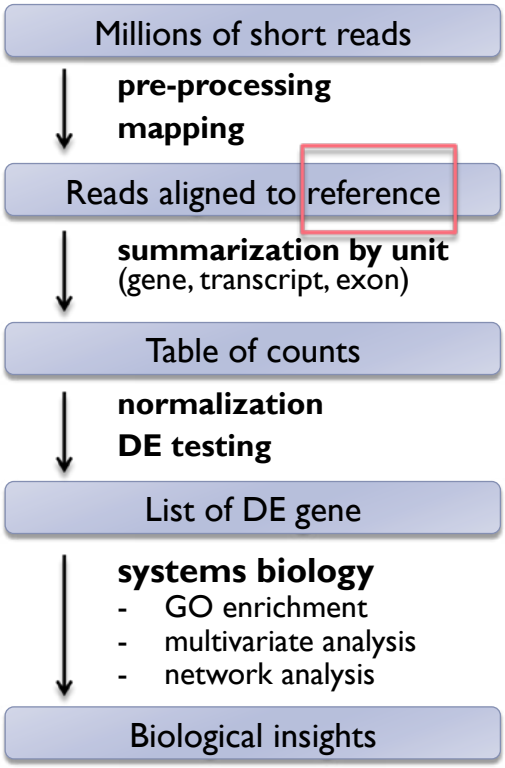
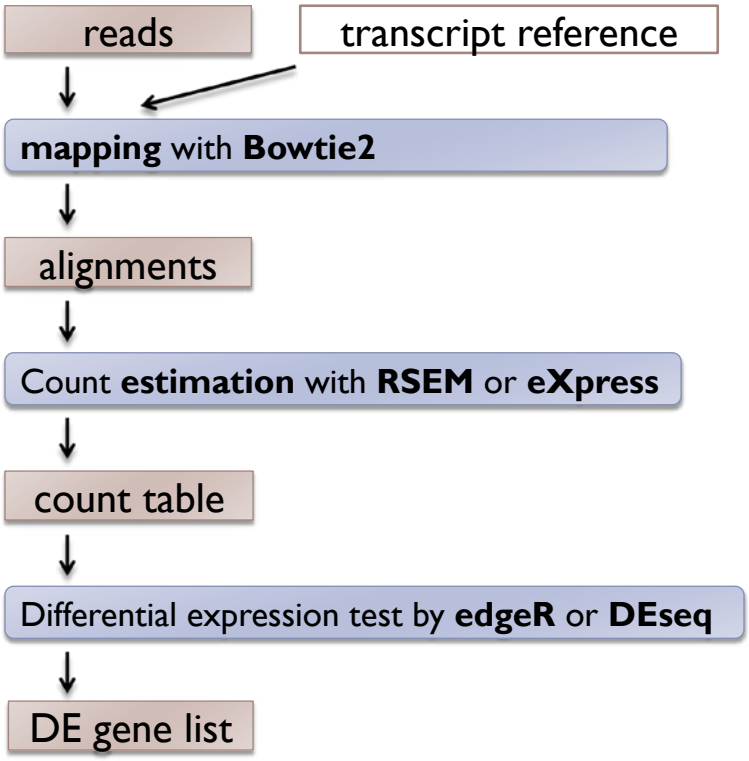
Two Basic Pipelines

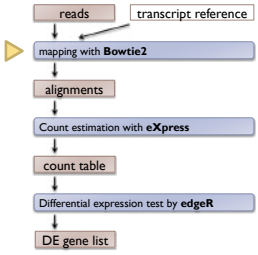
- Choice of reference
  - **Genome** – standard for genome-known species
  - **Transcript** – the only way for genome-unknown species
    -- can be used for genome-known species



A Pipeline: Transcript-based



Mapping – alignment software



- For mapping reads onto transcript reference

  short read mapper (unspliced read aligner) is used

  - **Bowtie2**

    http://bowtie-bio.sourceforge.net/bowtie2/index.shtml

bowtie2

Bowtie is an ultrafast, memory-efficient short read aligner.

http://bowtie-bio.sourceforge.net/bowtie2/index.shtml

```
(example)

$ bowtie2 –x transcript.fa –U reads.fq –a –S out.sam
```

- Input
  - Reads (fastq) and reference (bowtie2-db)
- Output
  - Alignment in SAM format : **`out.sam`**

Let's Try Bowtie2

Align 75-bp Illumina reads with a transcript reference using Bowtie2.

Prepare reads and reference genome

Sequences for this exercise are stored in `~/data/ss/`.

```
IlluminaReads1.fq – Illumina reads in fastq format
minimouse_mRNA.fa – a set of transcript sequnences
```

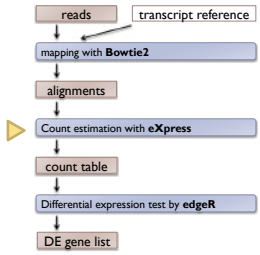
Build index of reference sequence

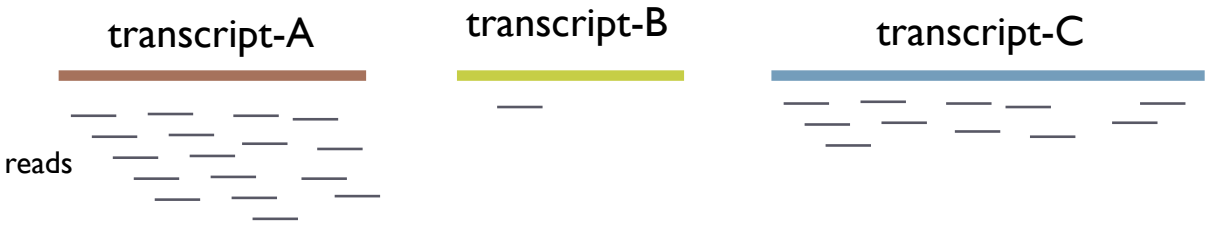
```
$bowtie2-build minimouse_mRNA.fa myref
```

Align reads with reference

```
$bowtie2 –x myref –U IlluminaReads1.fq –a –S out.sam
```

Count Reads by Transcript/gene





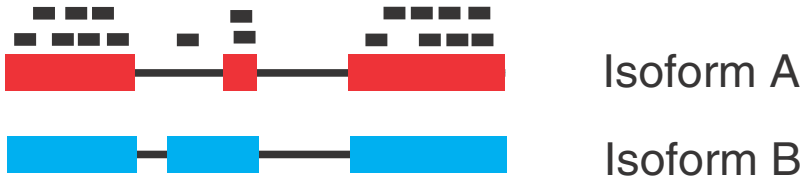
- The simplest way: just count reads by contig.

But…

- Mapping ambiguity should be taken into consideration.

Estimate Abundance

- **Multimapping issues**
  - Isoforms
  - Very similar paralogs
  - Repetitive sequences
  - => cannot align reads uniquely
- Mapping ambiguity should be taken into consideration.



- Critical for RNA-seq de novo analysis
- Software: RSEM and eXpress (EM algorithm)

eXpress

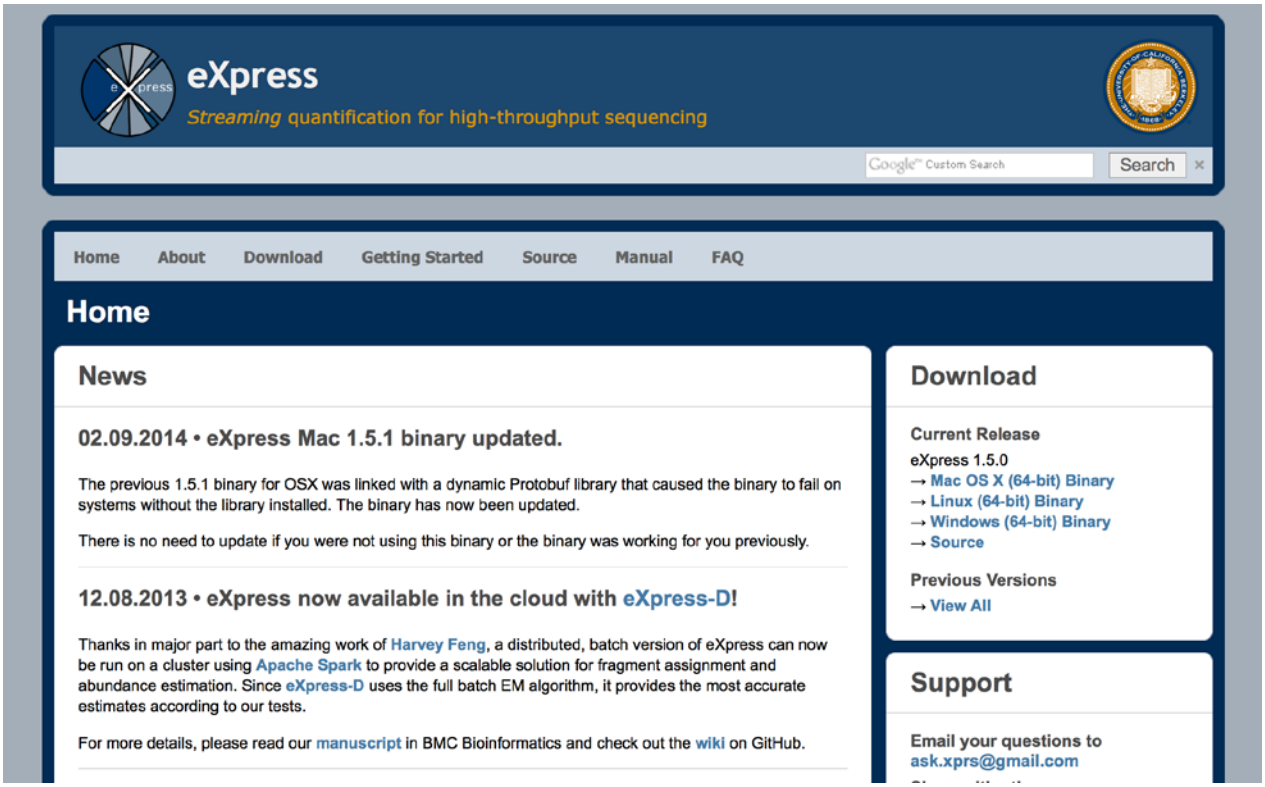
eXpress is a streaming tool for quantifying the abundances of a set of target sequences from sampled subsequences.

http://bio.math.berkeley.edu/eXpress/

```
(example)

$ express transcripts.fasta hits.bam
```

- Input
  - alignment (bam|sam) and reference (fasta)
- Output
  - Count estimation table: **`results.xprs`**



http://bio.math.berkeley.edu/eXpress/index.html

Let's Try eXpress

Prepare alignments and reference genome

Sequences for this exercise are stored in **~/data/SS/**.

```
IlluminaReads1.fq – Illumina reads in fastq format
out.sam – this file should be generated in the previous bowtie practice
```

Run eXpress

```
$ express minimouse_mRNA.fa out.sam
```

```
Output : results.xprs, params.xprs
```

eXpress: output

`results.xprs`

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	bundle_id	target_id	length	eff_length	tot_counts	uniq_counts	est_counts	eff_counts	ambig_distr_alpha	ambig_distr_beta	fpkm	fpkm_conf_low	fpkm_conf_high	solvable
2	1	m.245853	621	398.1	807	15	86.2	134.4	9.83E+01	9.96E+02	2.34E+01	1.88E+01	2.80E+01	T
3	1	m.245856	660	442.0	991	199	919.8	1373.4	5.53E+01	5.46E+00	2.25E+02	2.12E+02	2.38E+02	T
4	2	m.42076	1959	1591.7	156	156	156.0	192.0	0.00E+00	0.00E+00	1.06E+01	1.06E+01	1.06E+01	T
5	3	m.60782	291	83.0	12	12	12.0	42.1	0.00E+00	0.00E+00	1.57E+01	1.57E+01	1.57E+01	T
6	4	m.158451	282	64.5	0	0	0.0	0.0	0	0	0.00E+00	0.00E+00	0.00E+00	T
7	5	m.337354	219	39.4	0	0	0.0	0.0	0	0	0.00E+00	0.00E+00	0.00E+00	T
8	6	m.338934	261	82.3	0	0	0.0	0.0	0	0	0.00E+00	0.00E+00	0.00E+00	T
9	7	m.5973	822	719.9	4	4	4.0	4.6	0.00E+00	0.00E+00	6.01E-01	6.01E-01	6.01E-01	T
10	8	m.337793	219	38.7	0	0	0.0	0.0	0	0	0.00E+00	0.00E+00	0.00E+00	T
11	9	m.340910	210	40.5	0	0	0.0	0.0	0	0	0.00E+00	0.00E+00	0.00E+00	T
12	10	m.289784	3177	2521.4	350	350	350.0	441.0	0.00E+00	0.00E+00	1.50E+01	1.50E+01	1.50E+01	T
13	11	m.248666	240	61.8	1	1	1.0	3.9	0.00E+00	0.00E+00	1.75E+00	1.75E+00	1.75E+00	T
14	12	m.90727	240	55.7	13	13	13.0	56.1	0.00E+00	0.00E+00	2.53E+01	2.53E+01	2.53E+01	T
15	13	m.338727	216	48.1	0	0	0.0	0.0	0	0	0.00E+00	0.00E+00	0.00E+00	T
16	14	m.123519	225	43.2	0	0	0.0	0.0	0	0	0.00E+00	0.00E+00	0.00E+00	T
17	15	m.328661	251	50.8	1	1	1.0	4.9	0.00E+00	0.00E+00	2.13E+00	2.13E+00	2.13E+00	T
18	16	m.26062	642	356.1	1	1	1.0	1.8	0.00E+00	0.00E+00	3.04E-01	3.04E-01	3.04E-01	T
19	17	m.1295	240	53.6	0	0	0.0	0.0	0	0	0.00E+00	0.00E+00	0.00E+00	T
20	18	m.307626	201	220.2	4	3	3.0	2.7	8.33E+00	4.07E+04	1.47E+00	1.46E+00	1.49E+00	T
21	18	m.307625	204	35.7	301	300	301.0	1718.3	1.02E+01	2.10E-03	9.12E+02	9.05E+02	9.18E+02	T
22	19	m.49789	237	51.9	3	3	3.0	13.7	0.00E+00	0.00E+00	6.26E+00	6.26E+00	6.26E+00	T
23	20	m.33508	162	151.3	1	1	1.0	1.1	0.00E+00	0.00E+00	7.15E-01	7.15E-01	7.15E-01	T
24	21	m.109341	183	286.3	2	2	2.0	1.3	0.00E+00	0.00E+00	7.56E-01	7.56E-01	7.56E-01	T
25	22	m.331919	564	277.3	0	0	0.0	0.0	0	0	0.00E+00	0.00E+00	0.00E+00	T
26	23	m.23766	303	98.5	3	3	3.0	9.2	0.00E+00	0.00E+00	3.30E+00	3.30E+00	3.30E+00	T
27	24	m.246777	1149	1152.1	631	29	202.5	202.0	1.58E+02	3.90E+02	1.90E+01	1.65E+01	2.15E+01	T
28	24	m.246852	1323	1315.4	761	156	588.8	592.2	1.22E+02	4.85E+01	4.84E+01	4.50E+01	5.19E+01	T
29	24	m.246633	207	31.8	10	4	5.7	37.1	1.29E+04	3.27E+04	1.94E+01	1.05E+01	2.82E+01	T
30	24	m.246662	192	200.4	6	3	3.0	2.9	1.20E+01	3.22E+03	1.63E+00	1.51E+00	1.74E+00	T
31	25	m.99743	1641	1387.9	470	470	470.0	555.7	0.00E+00	0.00E+00	3.66E+01	3.66E+01	3.66E+01	T
32	26	m.335620	234	58.9	0	0	0.0	0.0	0	0	0.00E+00	0.00E+00	0.00E+00	T
33	27	m.16882	528	297.5	14	14	14.0	24.9	0.00E+00	0.00E+00	5.09E+00	5.09E+00	5.09E+00	T
34	28	m.77438	255	81.4	9	9	9.0	28.2	0.00E+00	0.00E+00	1.20E+01	1.20E+01	1.20E+01	T
35	29	m.131505	450	263.2	18	11	15.8	27.1	8.87E+00	3.95E+00	6.51E+00	4.68E+00	8.35E+00	T
36	29	m.131517	170	195.9	6	0	1.8	1.5	8.17E+00	1.96E+01	9.74E-01	0.00E+00	2.46E+00	T
37	29	m.131504	705	528.2	15	14	14.4	19.2	6.53E+01	1.01E+02	2.95E+00	2.69E+00	3.21E+00	T

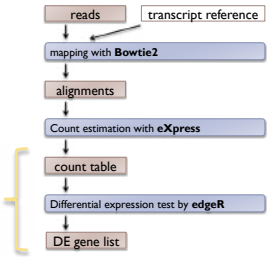


Table of counts

data import

diagnostics

normalization

DE testing

evaluation

List of DE gene

edgeR

- A Bioconductor package for differential expression analysis of digital gene expression data
- **Model**: An over dispersed Poisson model, negative binomial (NB) model, is used
- **Normalization**: TMM method (trimmed mean of M values) to deal with composition effects
- **DE test**: exact test and generalized linear models (GLM)

edgeR

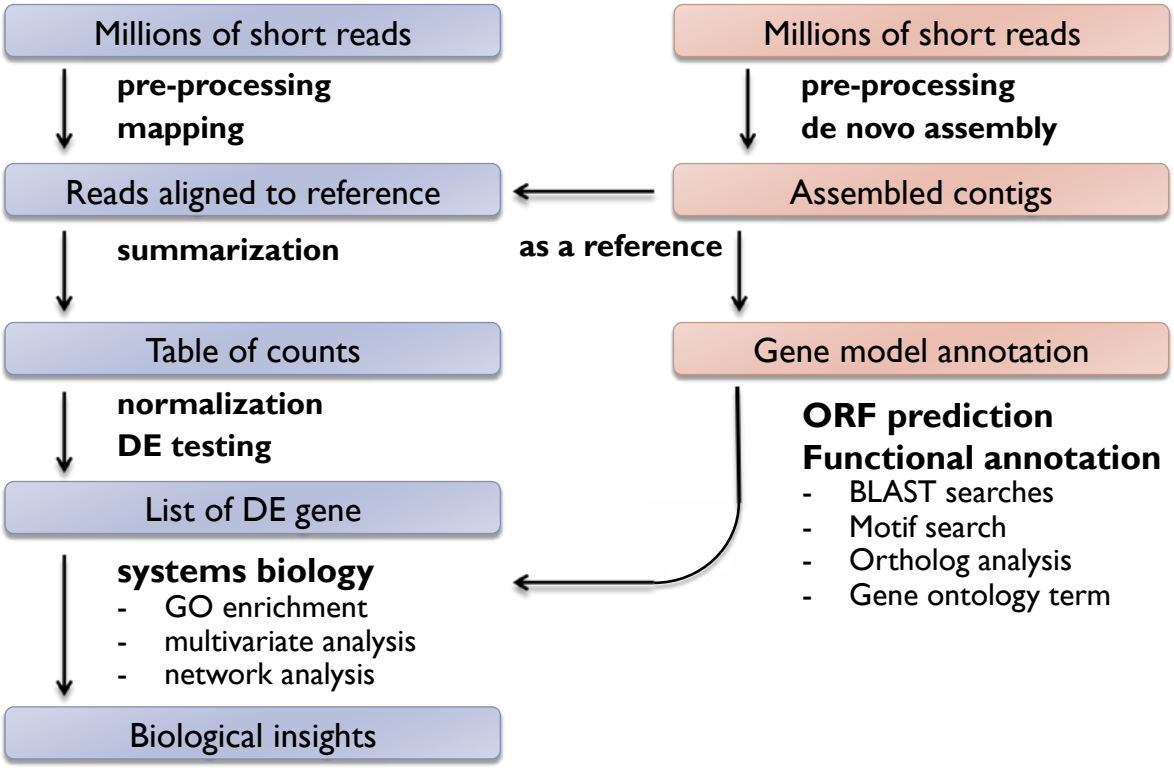
演習問題 ex5

- input: count data (not RPKM)
- output: gene table with DE significance statistics (FDR)

```
(example)

$ R
> library(edgeR)                        #load edgeR library
> dat <- read.delim("count_data.txt", …) #import count table to R
> group <- c(rep("M", 3), rep("H", 3))   #assign groups
> D <- DGEList(dat, group=group)         #import data to edgeR
> D <- calcNormFactors(D)                #normalization (TMM)
> D <- estimateCommonDisp(D)             #estimate common dispersion
> D <- estimateTagwiseDisp(D)            #estimate tagwise dispersion
> de <- exactTest(D, pair=c("M", "H")) #DE test
> topTags(de)
Comparison of groups: H-M
            logConc    logFC      P.Value          FDR
AT5G48430 -15.36821 6.255498 9.919041e-12 2.600872e-07
AT5G31702 -15.88641 5.662522 3.637593e-10 4.083773e-06
AT3G55150 -17.01537 5.870635 4.672331e-10 4.083773e-06
…
```

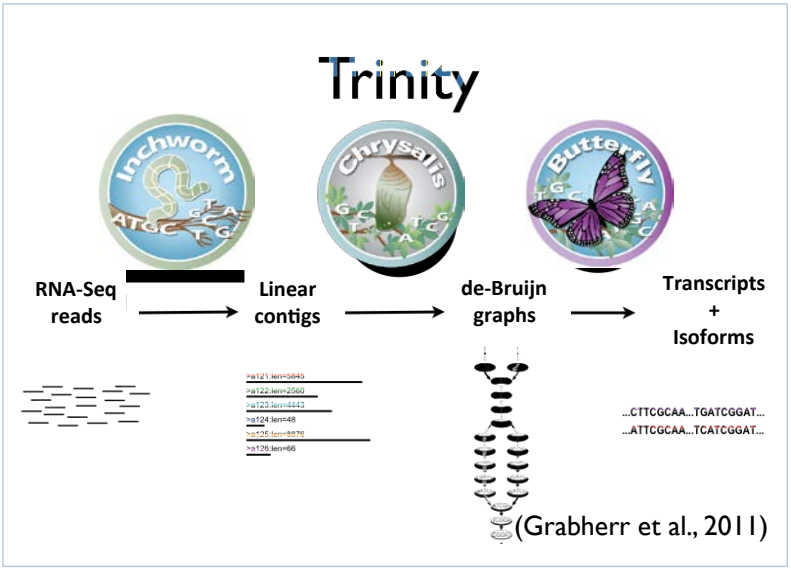
RNA-seq analysis pipeline (***de novo*** strategy)



de novo assemblers of RNA-seq

De novo assemblers use reads to assemble transcripts directly, which does not depend on a reference gnome.

- Trinity
- Oases
- TransAbyss
- EBARDenovo
- …



http://trinityrnaseq.sourceforge.net/

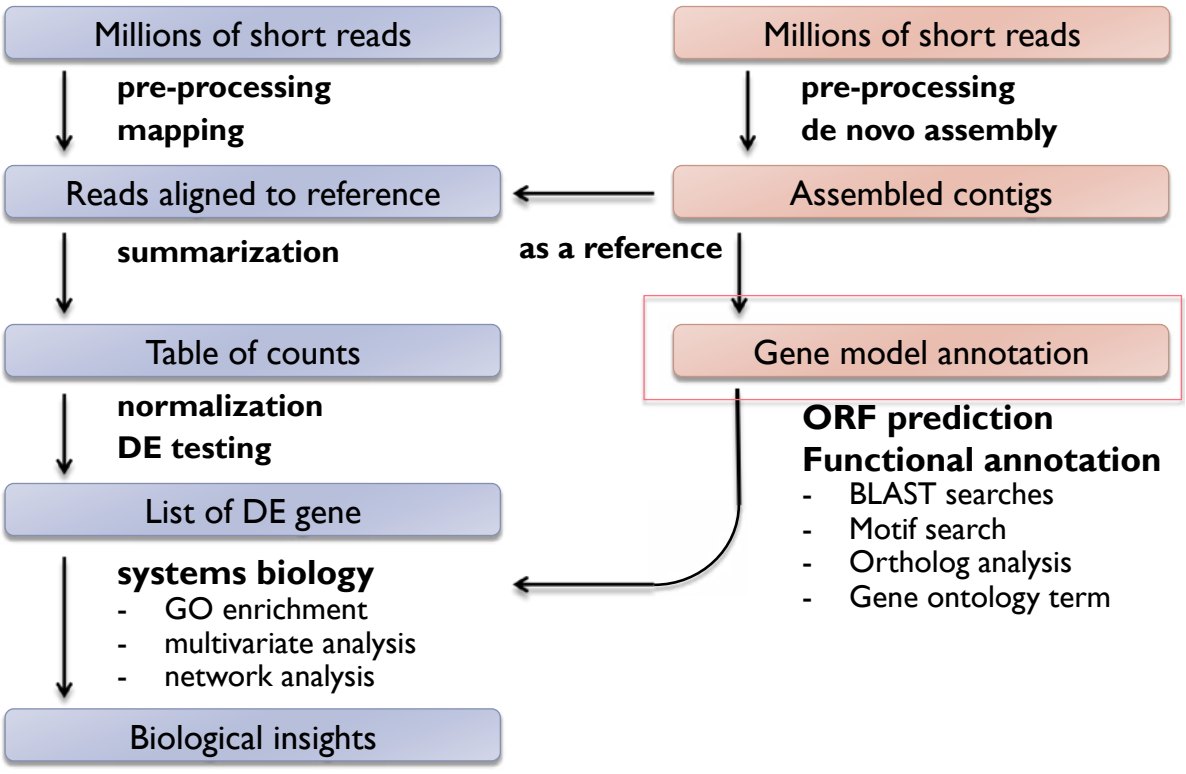
Trinity example

- Input: Illumina short reads in FASTQ | FASTA format
- Output: assembled contigs in FASTA format

```
# Run Trinity
$ Trinity --seqType fq --left left_all.fq --right right_all.fq \
          --CPU 8 --max_memory 20G
```

(Trinity is supported on only Linux)

RNA-seq analysis pipeline (*de novo* strategy)



optional

ORF prediction

- Special consideration in ORF prediction after de novo RNA-seq assembly
  - Sometimes partial: Start Met or terminal codon may be missing.
  - Ideally one ORF is present per contig, but erroneously joined contigs may include multiple ORFs.
  - Possible frame shifts.
    - Frame shifts do not occur so often in Illumina, while it happens very frequently in 454 and IonProton.
- Recommended software: TransDecoder

optional

Functional Annotation of Predicted ORFs

- BLAST
  - NCBI NR (or UniProt)
  - species of interest (model organisms, close relatives etc)
  - specific DB (SwissProt, rRNA DB, CEGMA etc)
  - self (assembly v.s. assembly)
- Motif search
  - Pfam, SignalP etc.
- Ortholog analysis
  - vs model organism
  - ortholog database (OrthoDB, eggNOG, OrthoMCL etc)
  - close relatives
- Gene Ontology term assignment

optional

Quick annotation by BLASTX

- Query: assembled contigs
  (nucleotide sequences in multi-fasta format)
- DB: Protein sequences of a model organism

Format DB

```
$ makeblastdb –in protein.fa -dbtype prot
```

Search

```
$ blastx -query trinity_contigs –db protein.fa \
  -num_threads 8 -evalue 1.0e-8 –outfmt 0 > blastxout.txt
```

optional

Let's try BLASTX

- Query: minimouse_mRNA.fa
- DB: human.protein.faa (human RefSeq protein)

1. Format DB

```
$ makeblastdb –in human.protein.faa -dbtype prot –parse_seqids
```

2. Search

```
$ blastx -query minimouse_mRNA.fa –db human.protein.faa \
  -num_threads 8 -evalue 1.0e-8 –outfmt 0 > blastxout.txt
```

```
$ blastx -query minimouse_mRNA.fa –db human.protein.faa \
  -num_threads 8 -evalue 1.0e-8 –outfmt 7 > blastxout.tab
```