RNA-Seq analysis in Galaxy

Advanced and alternative tools

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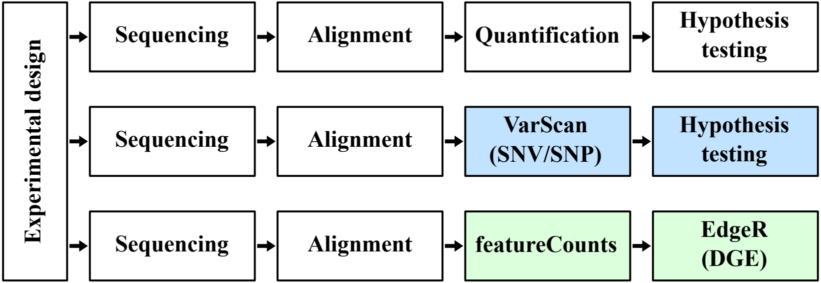
Overview

Introduction
- RNA-Seq

Single Nucleotide Variants (SNV)
- Introduction
- Tools
  - VarScan2
- Hands on

Differential Gene Expression (DGE) analysis
- Quantification
- Hypothesis testing
  - Sample pairing

RNA-seq analysis workflow(s)

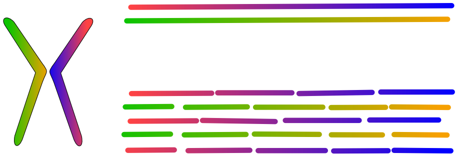


Single Nucleotide Variants

- 62,676,337 human SNPs in dbSNP (23-7-’13) [13]
- SNPs occur on average about every 100 to 300 bases (http://en.wikipedia.org/wiki/Human_genetic_variation)
- Contains information about heredity
- If expressed
  - Loss/change of protein
  - Loss of RNA 2D/3D structure
  - Affect alternative splicing

Major differences between SNVs in RNA-Seq & DNA-Seq

(allele specific) expression



Major differences between SNVs in RNA-Seq & DNA-Seq

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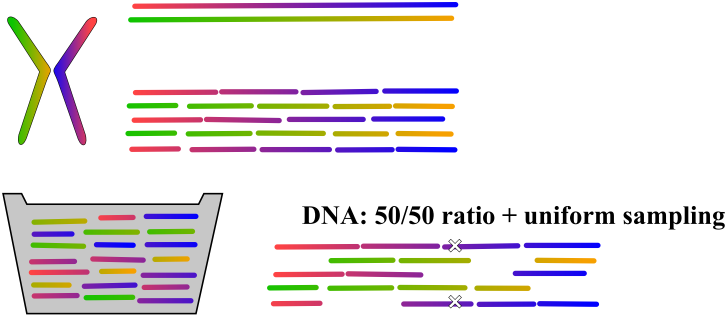
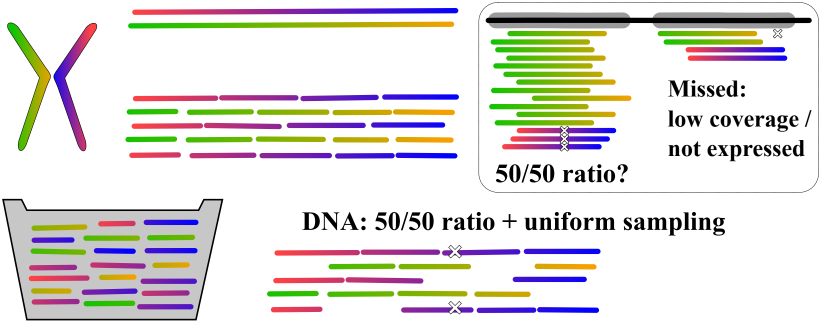


Major differences between SNVs in RNA-Seq & DNA-Seq

(allele specific) expression

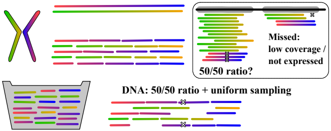


Missed:
low coverage /
not expressed

50/50 ratio?

DNA: 50/50 ratio + uniform sampling

Single Nucleotide Polymorphisms in RNA-Seq



- Major difference(s) between DNA-Seq:
  - Detected SNPs are expressed
    - Biological context
    - SNPs RNA-Seq only within exons and ncRNAs
    - Allele specific expression profiles
- Detection:
  - Expression affects coverage; in DNA-seq coverage should be uniform

Single Nucleotide Polymorphisms in RNA-Seq

Detection tools

- Alignment
  - TopHat [15, 5]
  - STAR [3]
  - ... many many more
- SNV calling
  - VarScan2 [6]
  - samtools [7]
  - exactSNP *(part of subread [9] package)*
  - GATK [17]

SNV detection in RNA using VarScan2

Requires samtools [7] for intermediate mpileup files

- Samtools: BAM file → mpileup file
- VarScan2
  - Compare alignment to reference genome
    - mpileup file + ref. fasta file → VCF file
    - Galaxy: *VarScan*
    - Galaxy: *VarScan ... optimized for direct BAM/SAM input*
  - Compare alignment to other alignment
    - 2×mpileup file → VCF file
    - Galaxy: *VarScan*
- *"statistical significance ... is computed by Fisher's exact test of the read counts supporting each allele (reference and variant) compared to the expected distribution based on sequencing error alone" [6]*

Single Nucleotide Polymorphisms in RNA-Seq

Using: Samtools, VarScan

reference	A	C	T	G	A	
read1	a	c	c	g	c	Alignment
read2	a	c	t	g	a	
read3	a	c	c	g	a	
read4	a	c	c	g	a	
read5	a	c	t	a	a	
read6		c	c	g	a	
read7			c	g	a	
read quality (q)	0.99	0.99	0.85	0.8	0.99	
aligned	5	6	7	7	7	Expected (based on quality)
q*aligned	4.95	5.94	5.95	5.6	6.93	
(1-q)*aligned	0.05	0.06	1.05	1.4	0.07	
exp match (abs)	5	6	6	6	7	
exp mismatch (abs)	0	0	1	1	0	
obs match	5	6	2	6	6	Observed
obs mismatch	0	0	5	1	1	
P(obs|exp) fisher exact	1.000	1.000	0.049	0.538	0.500	Hypothesis testing
P < 0.05	REF	REF	SNP	REF	REF	

Single Nucleotide Polymorphisms in RNA-Seq

Covered examples during hands-on

- SNP: Artificial alignment (hg19)
- InDel: MCF7 alignment (hg18) [10, 1]

Differential gene expression

- Triggered by
  - Stimuli (signal molecules)
  - Genetics (mutation)
- Genes interact in a network, effect spreads out
  - Genetic redundancy / biological robustness (often multiple changes necessary to cause a disease)

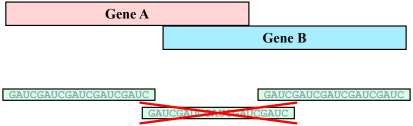
Differential gene expression analysis tools

- Alignment
  - TopHat [15, 5]
  - STAR [3]
  - ... many many more
- Measuring expression (quantification)
  - HTSeq-count [2]
  - Cufflinks [16]
  - featureCounts [8]
- Group-wise comparison (hypothesis testing)
  - EdgeR [12]
  - DESeq2 [11]
  - Cuffdiff [14]

Measure expression levels in RNA-Seq data

1. Align read to reference genome
2. Measuring expression = counting aligned reads
   - Count in annotated exons
   - Positive integers (read counts of 3.1415 or −42 are impossible)
   - Quantitative (read count has an absolute meaning)

- Observation (read count) must be statistically independent
  - No multi-map reads
  - Skip overlapping gene annotations



Measure expression levels in RNA-Seq data

In Galaxy

- featureCounts [8]
  - Pro's
    - Fast
    - Flexible
    - Free (GPL)
    - Accepts both BAM and SAM files
    - Only requires name-sorted files when mate-pairs are counted together (name-sorting is slow)
  - Con's
    - Built-in name-sorting supports no threading – rather do this with samtools [7]

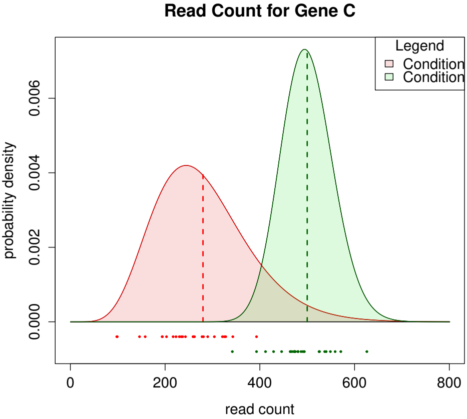
Differential gene expression analysis

edgeR

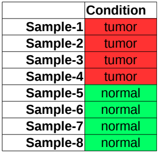
- edgeR [12]
  - Differential gene expression analysis
  - Free R Package (GPL2)
  - Galaxy wrapper does normalizations for you
    - Use raw reads, do NOT use FPKM/RPKM!
- "Limma" for count data
  - Not Gaussian (normal) distributed like e.g. micro-array data – but negative binomial

Differential gene expression analysis

Read counts: negative binomial distributed

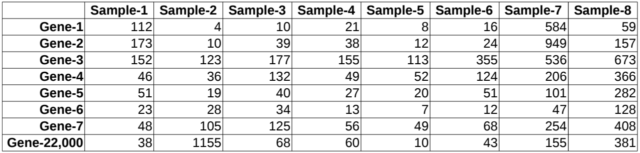


Differential gene expression analysis



	Condition
Sample-1	tumor
Sample-2	tumor
Sample-3	tumor
Sample-4	tumor
Sample-5	normal
Sample-6	normal
Sample-7	normal
Sample-8	normal

Design matrix



	Sample-1	Sample-2	Sample-3	Sample-4	Sample-5	Sample-6	Sample-7	Sample-8
Gene-1	112	4	10	21	8	16	584	59
Gene-2	173	10	39	38	12	24	949	157
Gene-3	152	123	177	155	113	355	536	673
Gene-4	46	36	132	49	52	124	206	366
Gene-5	51	19	40	27	20	51	101	282
Gene-6	23	28	34	13	7	12	47	128
Gene-7	48	105	125	56	49	68	254	408
Gene-22,000	38	1155	68	60	10	43	155	381

Expression matrix

Differential gene expression analysis

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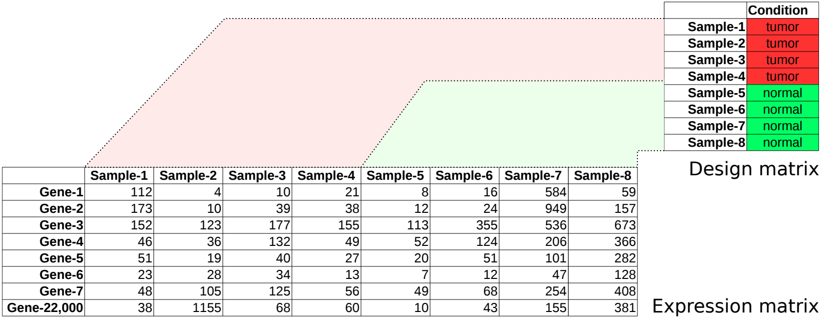

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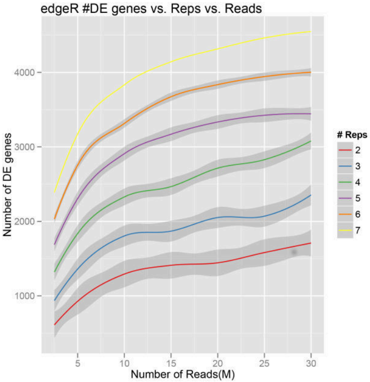
Expression matrix

Contrast = tumor ↔ normal

Differential gene expression analysis

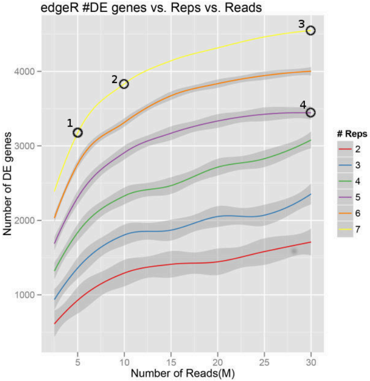
MCF7 cell line



[10]

Differential gene expression analysis

MCF7 cell line



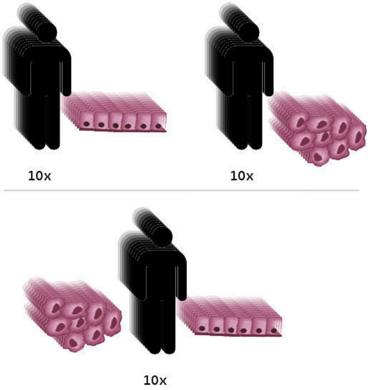
[10]

Differential gene expression analysis: sample pairing

- Sample pairing (do not confuse with PE-reads!) / batch effects
- Goal: correction for patient / batch specific expression profiles
- Examples:
    - 10×Tumour & Normal (both of same patient)
    - 3 populations: African, American & Asian
    - 2 batches: 1 at Monday, 1 at Friday

Differential gene expression analysis

Prostate cancer and normal prostate



[4]

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[8] Yang Liao, Gordon K Smyth, and Wei Shi. featurecounts: an efficient general-purpose program for assigning sequence reads to genomic features. *Bioinformatics*, 2013.

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[12] Mark D. Robinson, Davis J. McCarthy, and Gordon K. Smyth. edger: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, 26(1):139–140, 2010.

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More links

- http://www.bioinformatics.babraham.ac.uk/training/RNA-Seq_analysis_course.pptx
- http://galaxy.ctmm-trait.nl/
- http://toolshed.dtls.nl/