RNA-Seq Tutorial 1

John Garbe
Research Informatics Support Systems, MSI
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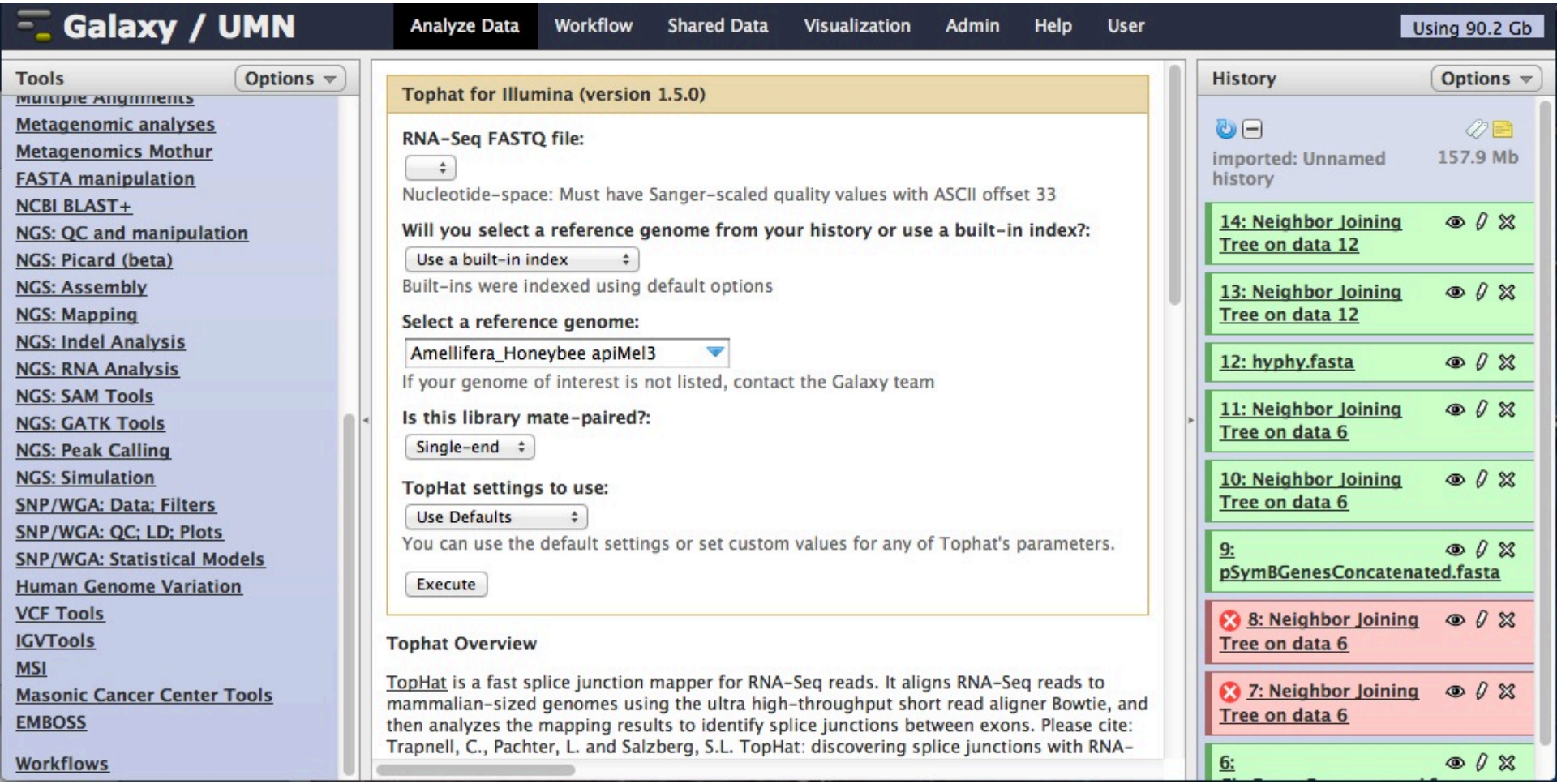
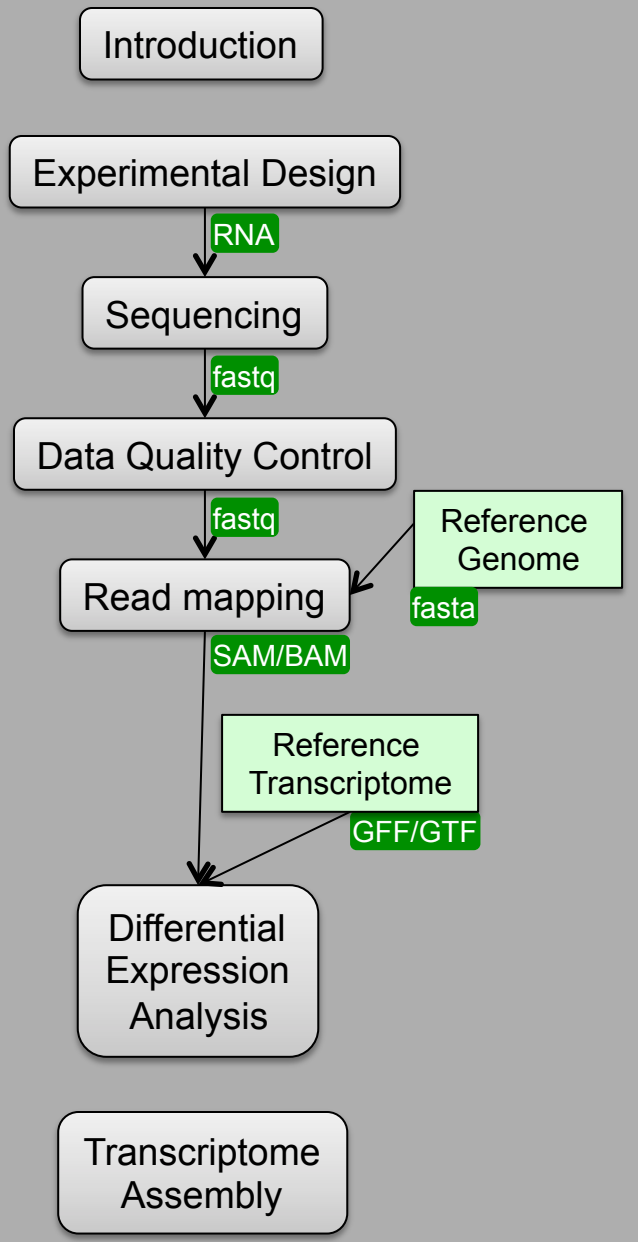
RNA-Seq Tutorials

- Tutorial 1
  - RNA-Seq experiment design and analysis
  - Instruction on individual software will be provided in other tutorials
- Tutorial 2
  - Advanced RNA-Seq Analysis topics
- Hands-on tutorials
  - Analyzing human and potato RNA-Seq data using Tophat and Cufflinks in Galaxy

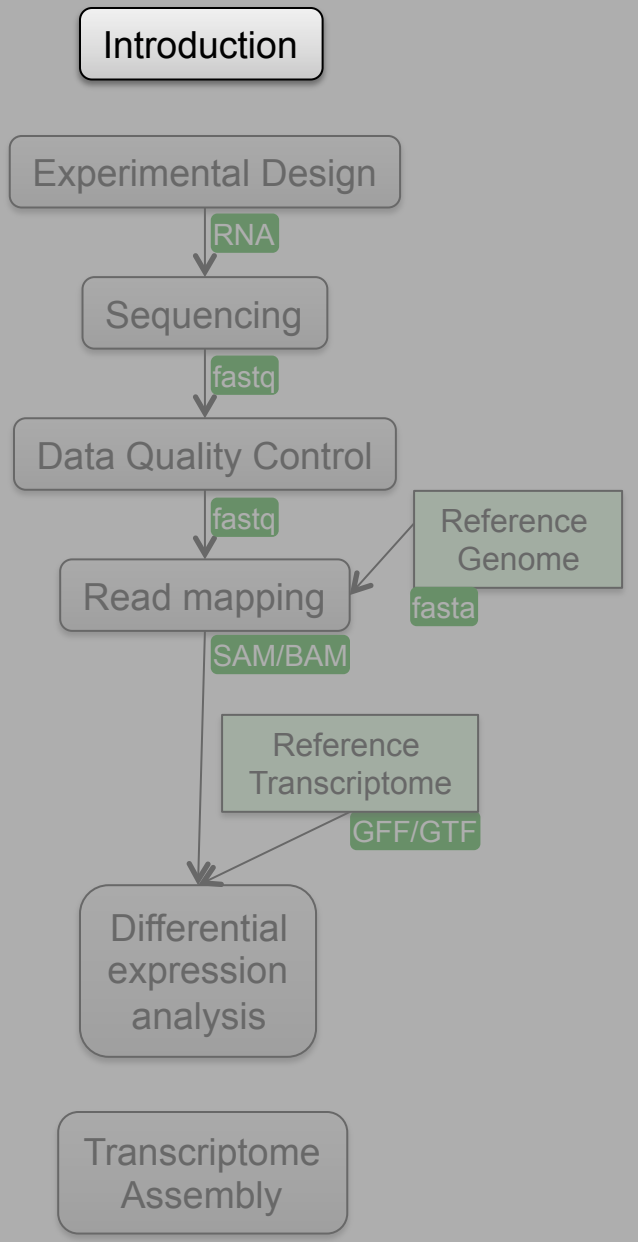
Galaxy.msi.umn.edu



Web-based platform for bioinformatic analysis


Introduction
Experimental Design
RNA
Sequencing
fastq
Data Quality Control
fastq
Reference Genome
fasta
Read mapping
SAM/BAM
Reference Transcriptome
GFF/GTF
Differential Expression Analysis
Transcriptome Assembly

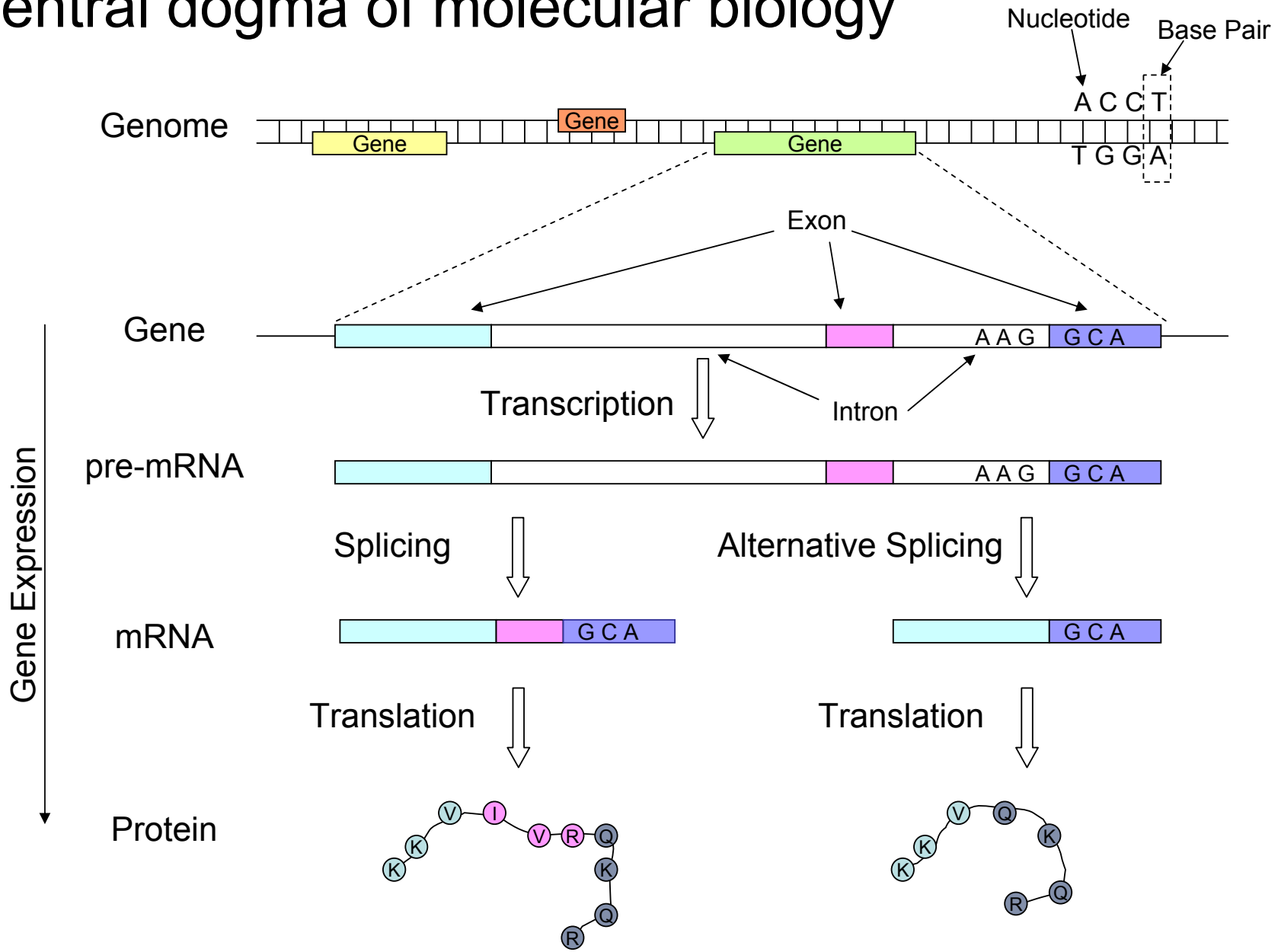
Outline



Introduction

- Gene expression
- RNA-Seq
- Platform characteristics
- Microarray comparison

Central dogma of molecular biology

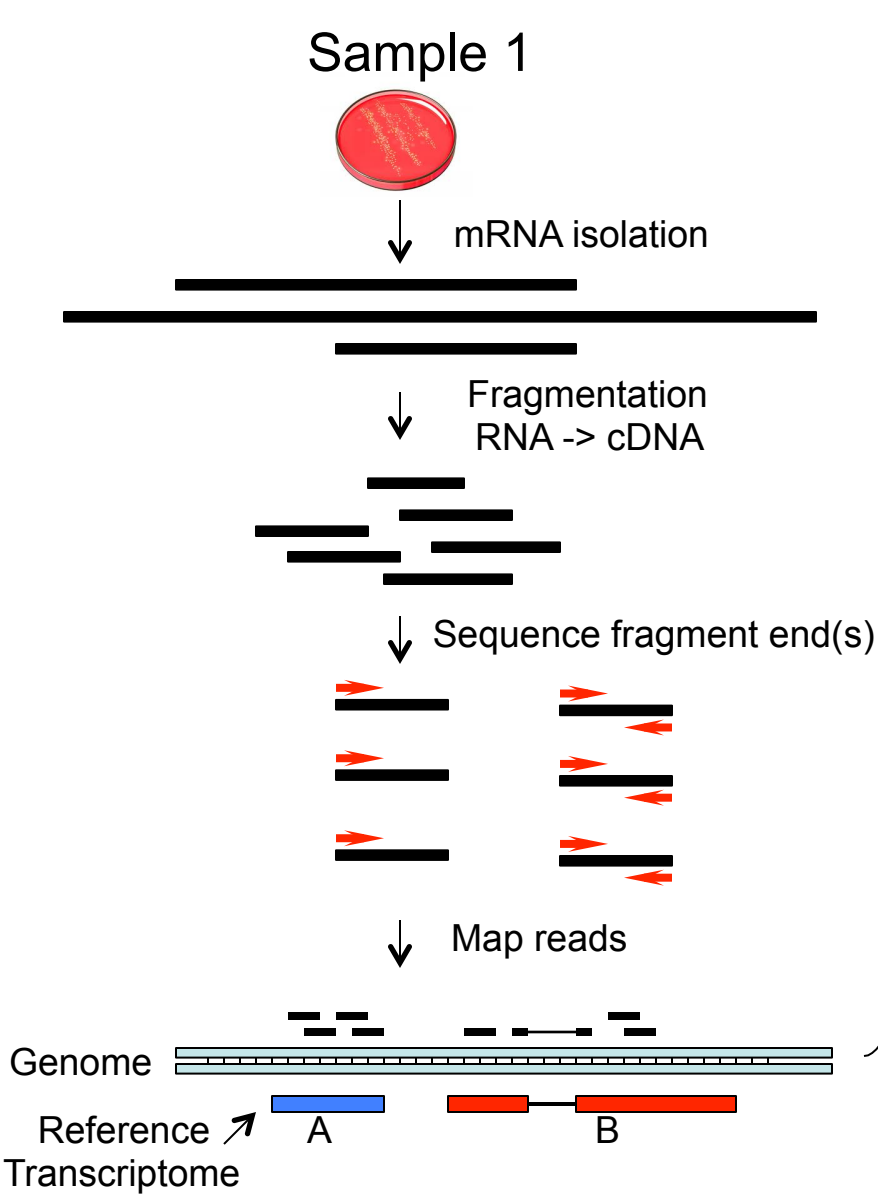


92–94% of human genes undergo alternative splicing,
86% with a minor isoform frequency of 15% or more

E.T. Wang, et al, Nature 456, 470-476 (2008)

Introduction

- RNA-Seq
  - High-throughput sequencing of RNA
  - Transcriptome assembly
    - Qualitative identification of expressed sequence
  - Differential expression analysis
    - Quantitative measurement of transcript expression



Calculate transcript abundance

	Gene A	Gene B
Sample 1	4	4

# of Reads

	Gene A	Gene B
Sample 1	4	2

Reads per kilobase of exon

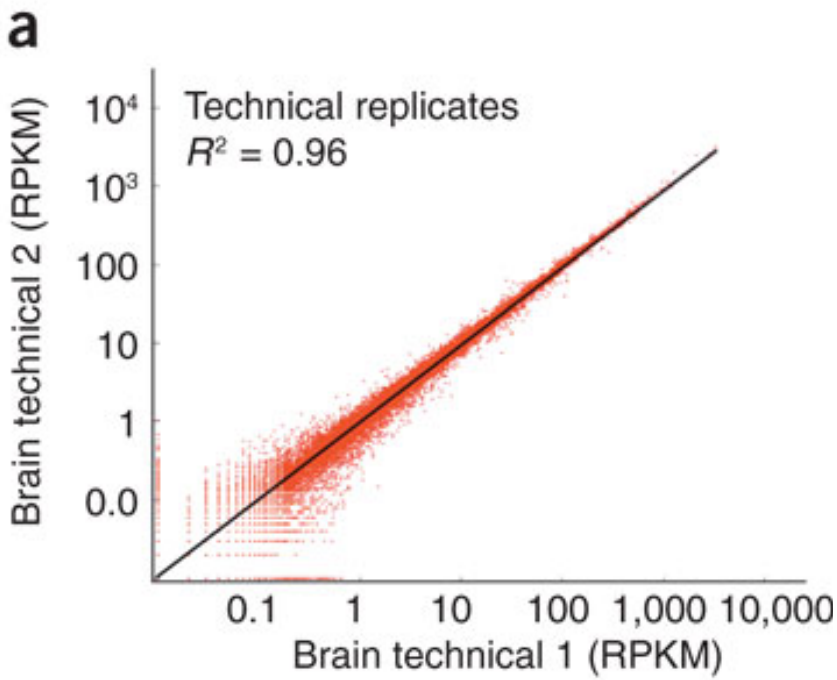
	Gene A	Gene B	Total
Sample 1	4	2	6
Sample 2	7	5	12

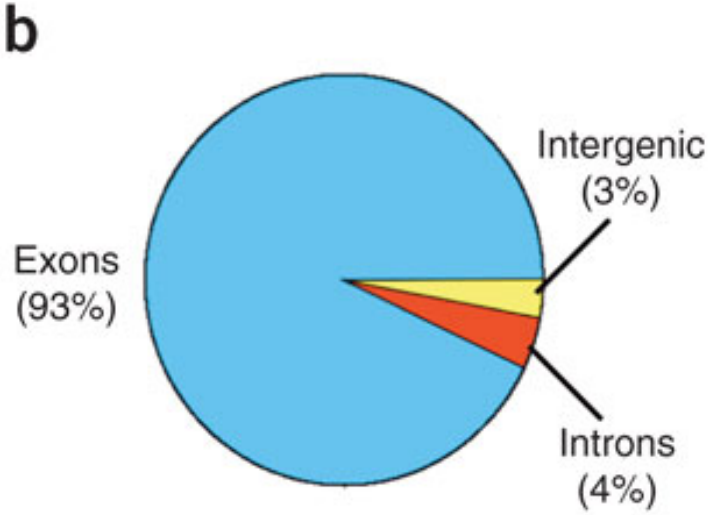
Reads per kilobase of exon

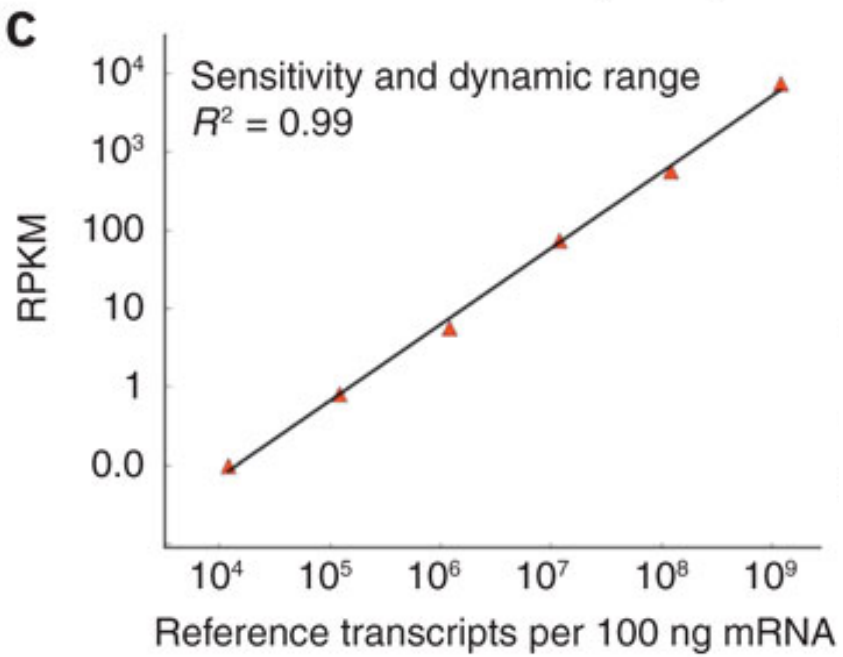
	Gene A	Gene B	Total
Sample 1	.7	.3	6
Sample 2	.6	.3	12

Reads per kilobase of exon per million mapped reads

RPKM



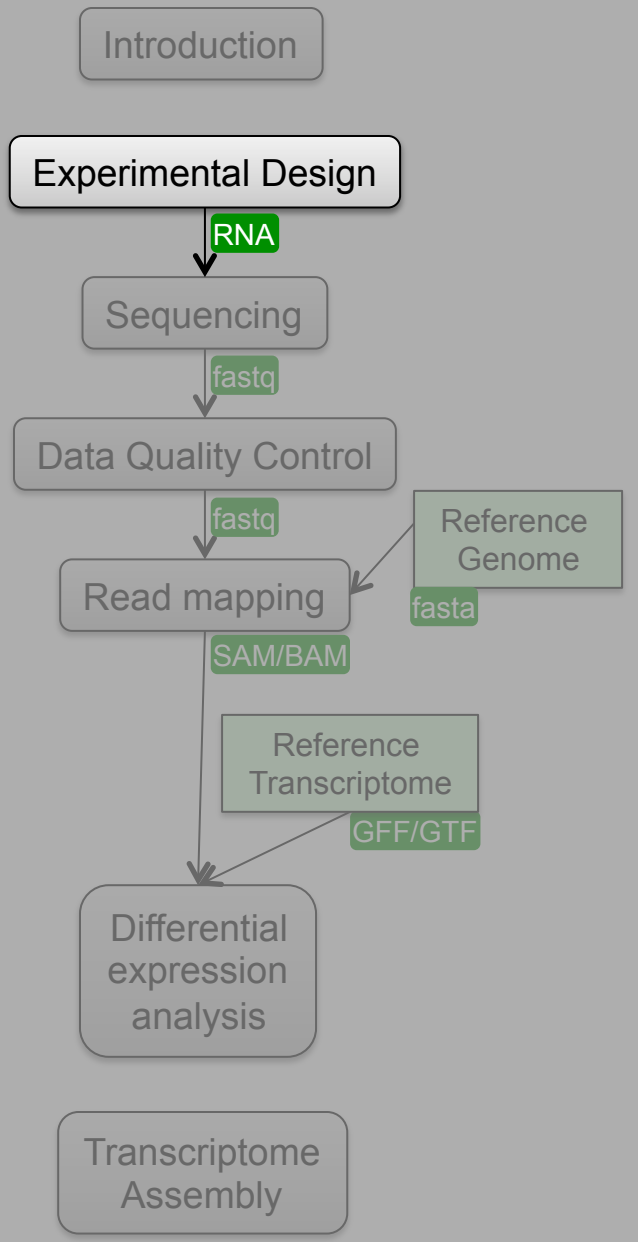




Ali Mortazavi et al., *Nature Methods* - **5**, 621 - 628 (2008)

Introduction

- RNA-Seq (vs Microarray)
  - Strong concordance between platforms
  - Higher sensitivity and dynamic range
  - Lower technical variation
  - Available for all species
  - Novel transcribed regions
  - Alternative splicing
  - Allele-specific expression
  - Fusion genes
  - Higher informatics cost

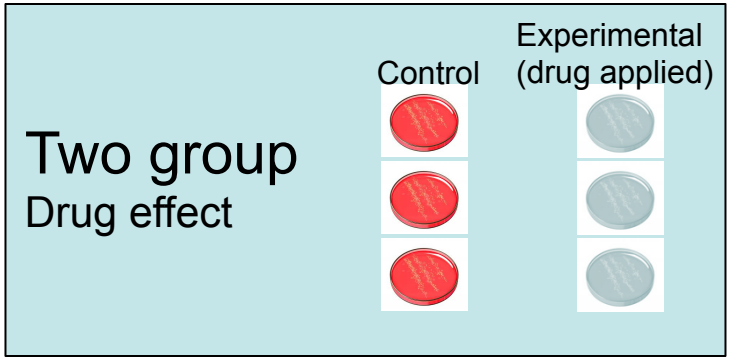


Experimental Design

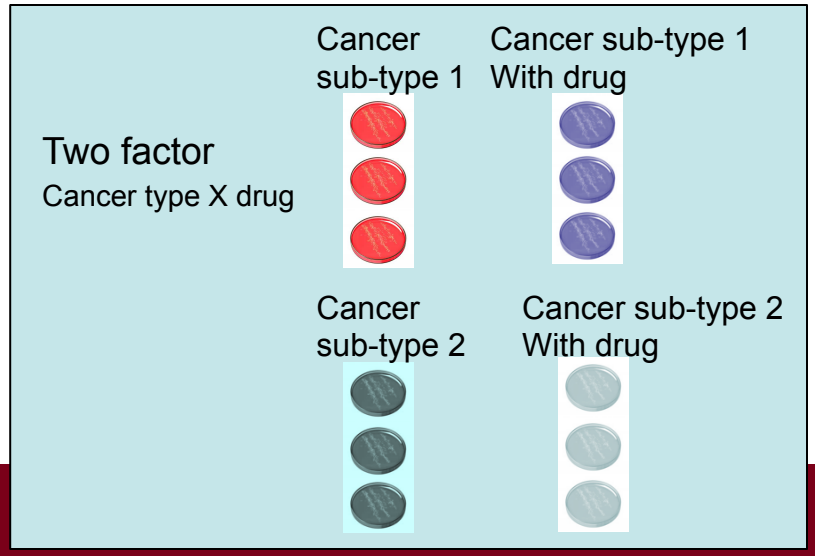
- Biological comparison(s)
- Paired-end vs single end reads
- Read length
- Read depth
- Replicates
- Pooling

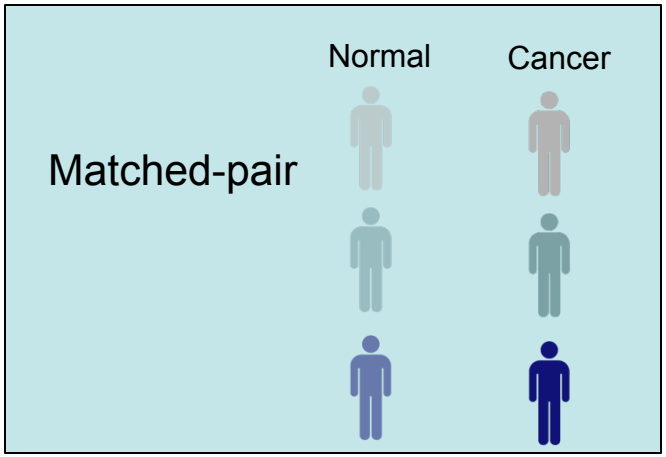
Experimental design

- Simple designs (Pairwise comparisons)



- Complex designs – Consult a statistician





Experimental design

- What are my goals?
  - Transcriptome assembly?
  - Differential expression analysis?
  - Identify rare transcripts?
- What are the characteristics of my system?
  - Large, complex genome?
  - Introns and high degree of alternative splicing?
  - No reference genome or transcriptome?

Experimental design

HiSeq 2000 Rates	10 million reads (1/16 lane)	20 million reads (1/8 lane)	40 million reads (1/4 lane)	80 million reads (1/2 lane)	160 million reads (1 lane)
Single-read (1x50 cycles)	$266	$331	$462	$724	$1,249
Single-read (1x100 cycles)	$282	$364	$528	$857	$1,515
Paired-end read (2x50 cycles)	$297	$394	$589	$979	$1,758
Paired-end (2x100 cycles)	$328	$456	$712	$1,225	$2,251

UMGC RNA-Seq Price list

-Valid through June 30, 2013
-Volume discounts available

Experimental design

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10 million reads per sample, 50bp single-end reads

- Small genomes with no alternative splicing

Experimental design

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20 million reads per sample, 50bp paired-end reads

- Mammalian genomes (large transcriptome, alternative splicing, gene duplication)

Experimental design

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40-160 million reads per sample, 100bp paired-end reads

- Transcriptome Assembly (100X coverage of transcriptome)

50bp Paired-end >> 100bp Single-end

Experimental design

- Technical replicates
  - Not needed: low technical variation
    - Minimize batch effects
    - Randomize sample order 
- Biological replicates
  - Not needed for transcriptome assembly
  - Essential for differential expression analysis
  - Difficult to estimate
    - 3+ for cell lines
    - 5+ for inbred lines
    - 20+ for human samples

Experimental design

- Pooling samples
  - Limited RNA obtainable
    - Multiple pools per group required
  - Transcriptome assembly

Experimental design

RNA-seq: technical variability and sampling

Lauren M McIntyre, Kenneth K Lopiano, Alison M Morse, Victor Amin, Ann L Oberg, Linda J Young and Sergey V Nuzhdin

BMC Genomics 2011, 12:293

Statistical Design and Analysis of RNA Sequencing Data

Paul L. Auer and R. W. Doerge

Genetics. 2010 June; 185(2): 405–416.

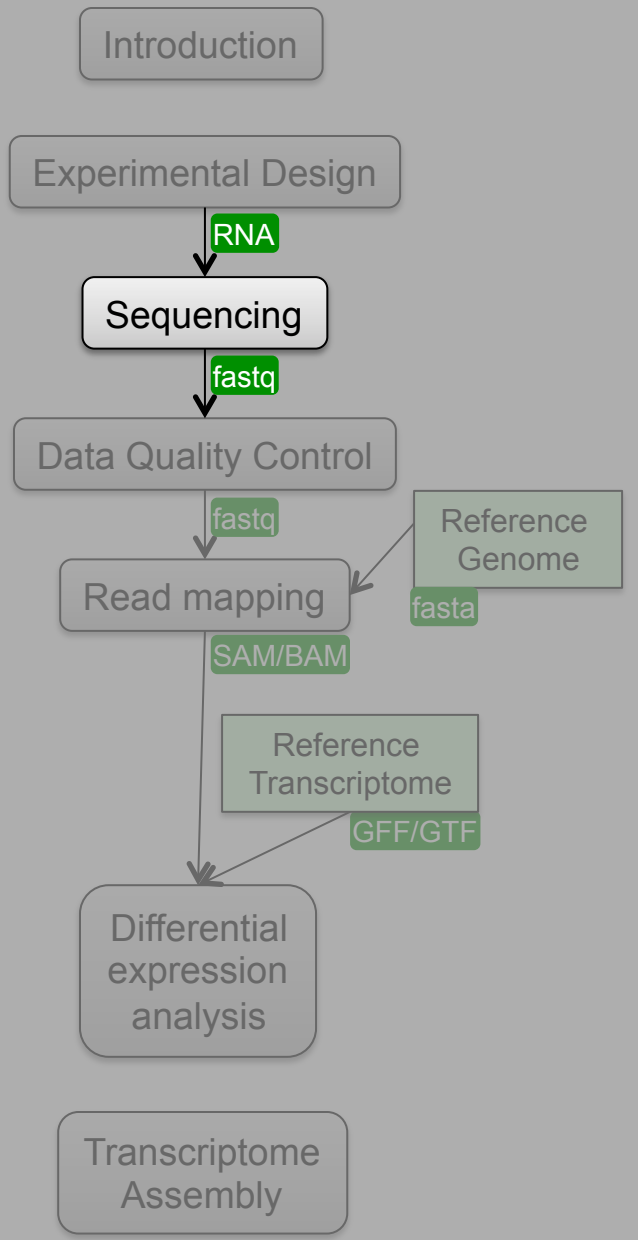
Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries

Daniel Aird, Michael G Ross, Wei-Sheng Chen, Maxwell Danielsson, Timothy Fennell, Carsten Russ, David B Jaffe, Chad Nusbaum and Andreas Gnirke

Genome Biology 2011, 12:R18

ENCODE RNA-Seq guidelines

http://www.encodeproject.org/ENCODE/experiment_guidelines.html



Sequencing

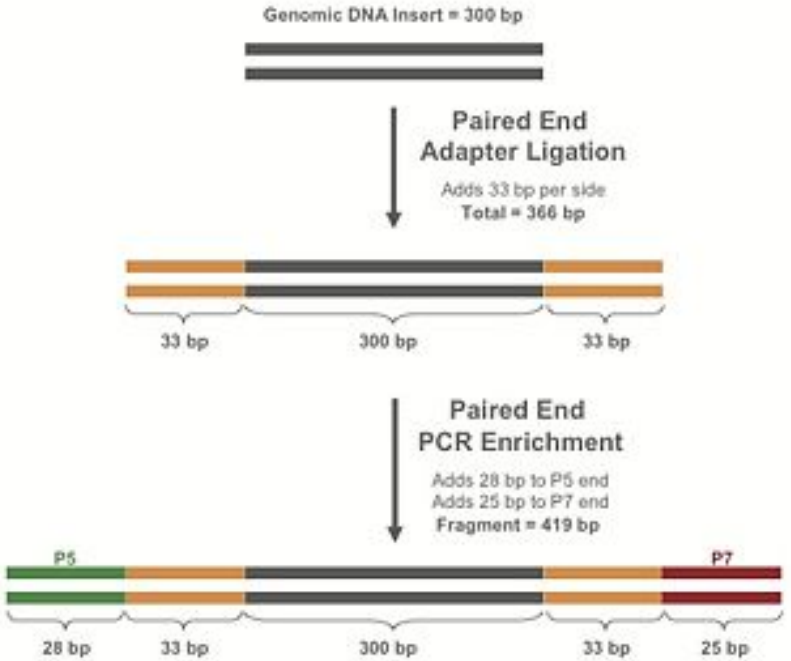
- Platforms
- Library preparation
- Multiplexing
- Sequence reads

Sequencing

- Illumina sequencing by synthesis
  - GAIIx
    - replaced by HiSeq
  - HiSeq2000 (dominant RNA-Seq platform) (HiSeq2500 coming soon)
  - MiSeq
    - low throughput, longer reads (2x250), fast turnaround
- SOLiD (not available at UMGC)
  - “Color-space” reads (require special mapping software)
  - Low error rate
- 454 pyrosequencing
  - Longer reads, lower throughput, high cost

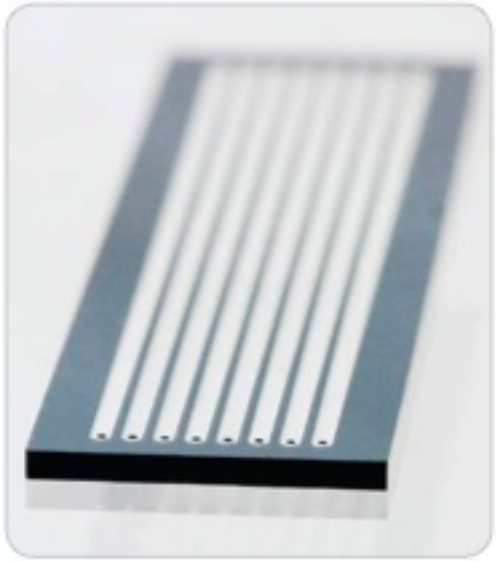
Sequencing

- Library preparation (Illumina TruSeq protocol for HiSeq)
  - RNA isolation
  - Ploy-A purification
  - Fragmentation
  - cDNA synthesis using random primers
  - Adapter ligation
  - Size selection
  - PCR amplification

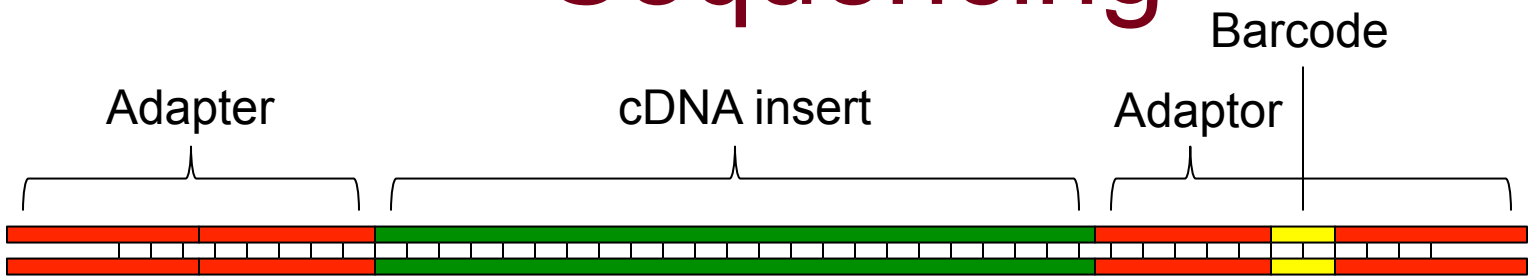


Sequencing

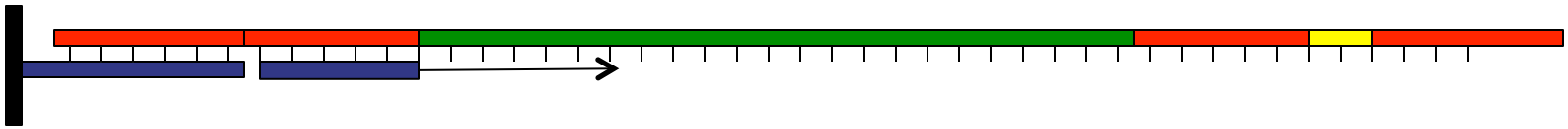
- Flowcell
  - 8 lanes
  - 160+ Million reads per lane
  - Multiplex up to 8 samples on one lane using barcodes



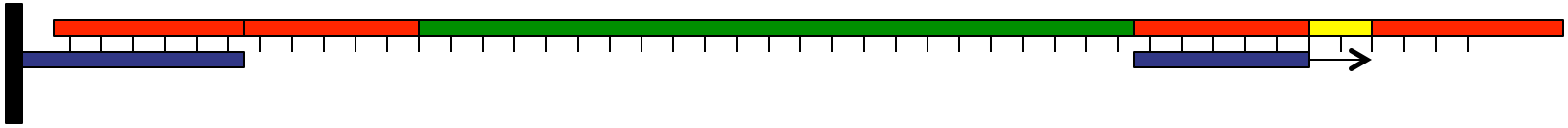
Sequencing



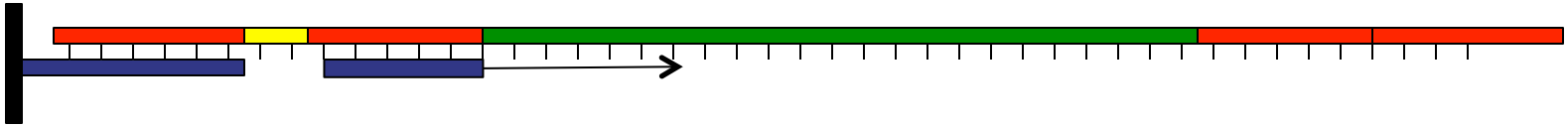
Read 1
"left" read



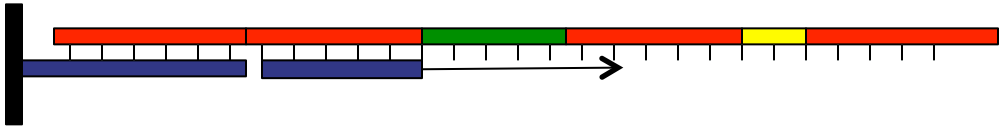
Index read



Read 2
"right" read
(optional)



Adaptor
contamination

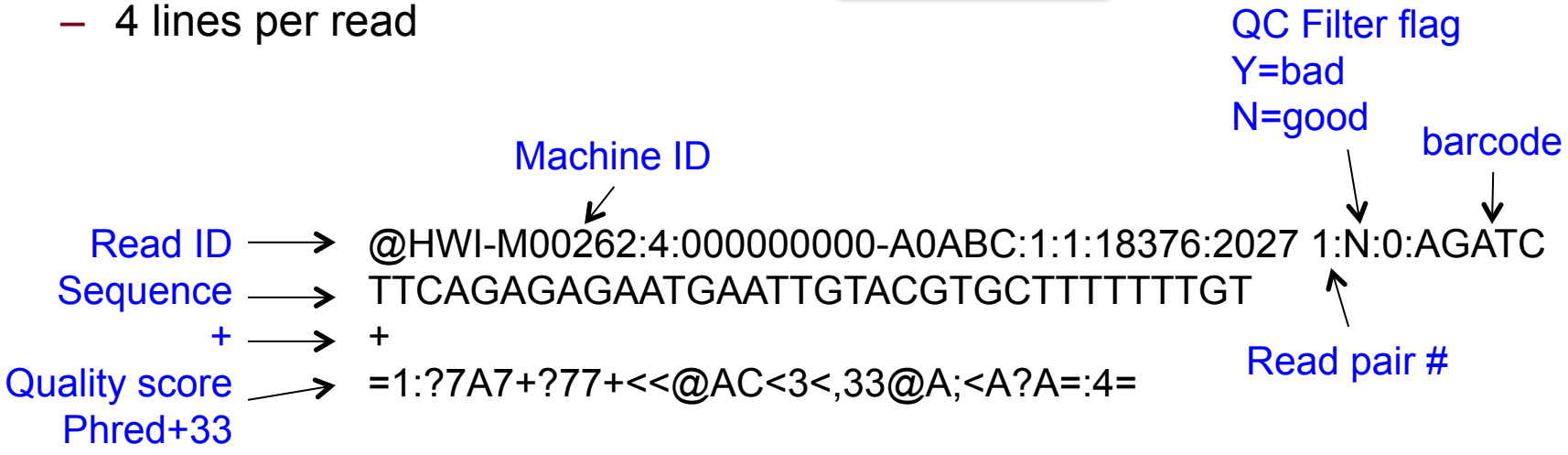


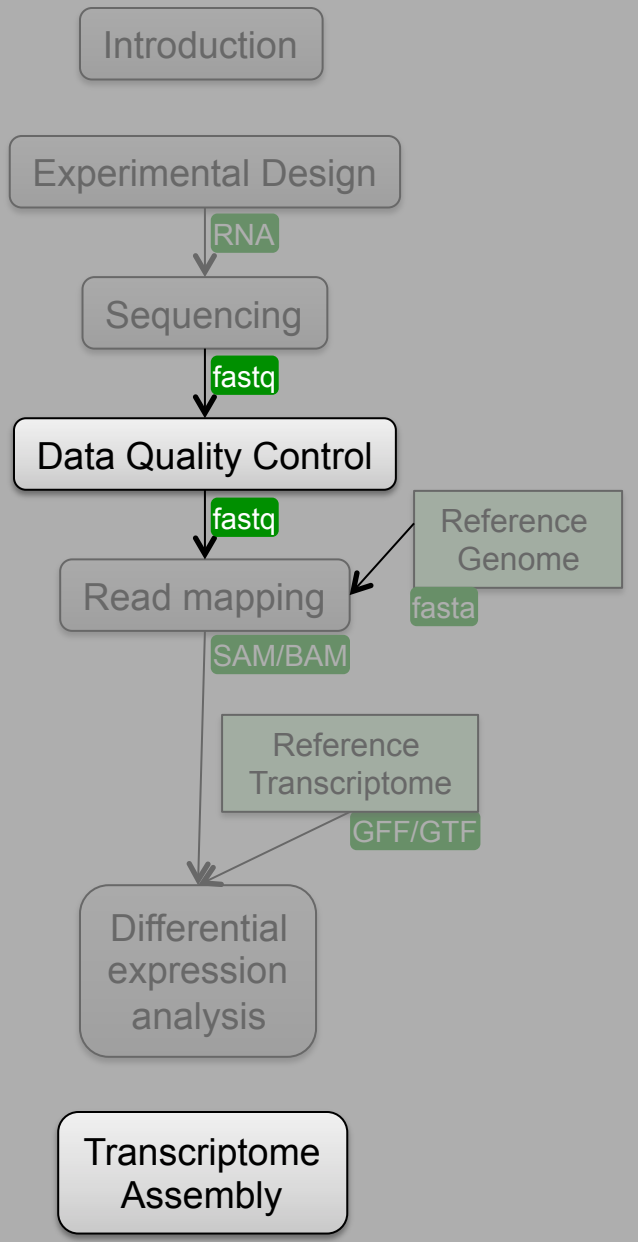
Sequencing

- Library types
  - Polyadenylated RNA > 200bp (standard method)
  - Total RNA
  - Small RNA
  - Strand-specific
    - Gene-dense genomes (bacteria, archaea, lower eukaryotes)
    - Antisense transcription (higher eukaryotes)
  - Low input
  - Library capture

Sequence Data Format

- Data delivery
  - /home/PI-groupname/data_release/umgc/hiseq/120318_SN261_0348_A81JUMABXX
  - Upload to Galaxy
- File names
  - L1_R1_CCAAT_cancer1.fastq
  - L1_R2_CCAAT_cancer1.fastq
- Fastq format (Illumina Casava 1.8.0)— Formats vary
  - 4 lines per read





Data Quality Control

- Quality assessment
- Trimming and filtering

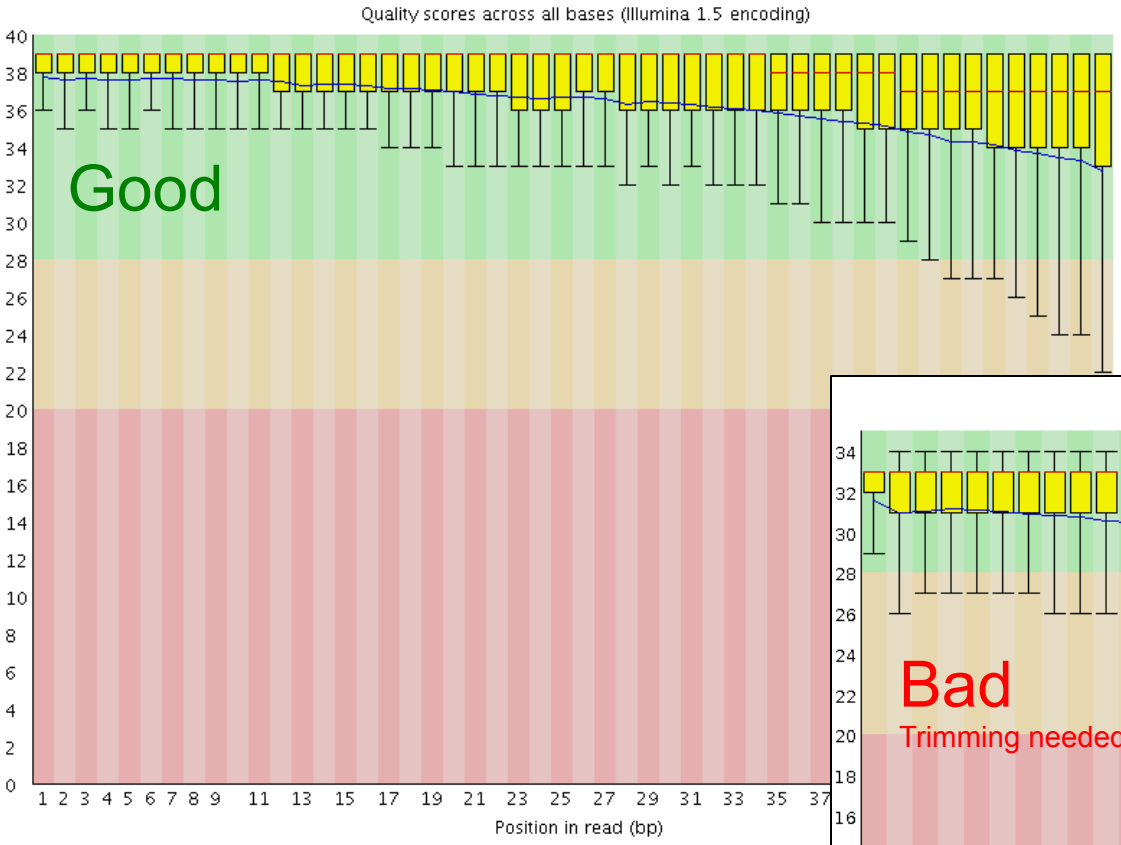
Data Quality Assessment

- Evaluate read library quality
  - Identify poor/bad samples
  - Identify contaminants
- Software
  - FastQC (recommended)
    - Command-line, Java GUI, or Galaxy
  - SolexaQC
    - Command-line
    - Supports quality-based read trimming and filtering
  - SAMStat
    - Command-line
    - Also works with bam alignment files

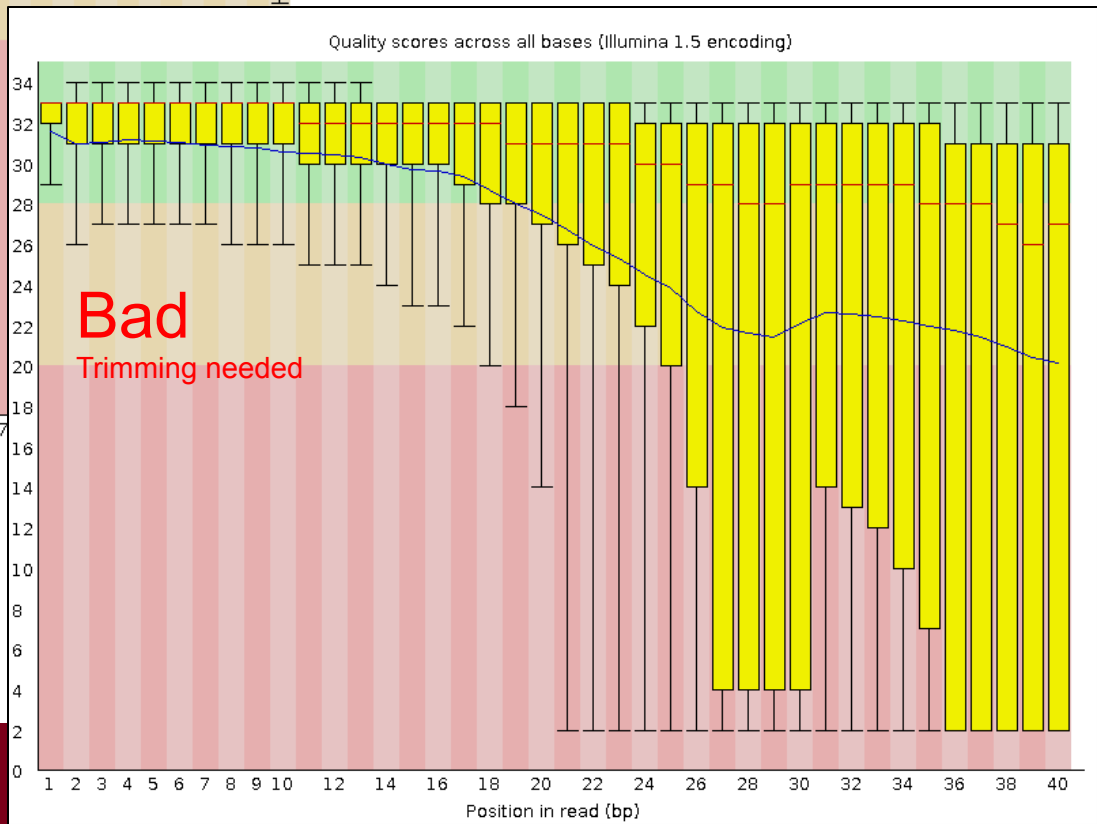
Data Quality Assessment

- Trimming: remove bad bases from (end of) read
  - Adaptor sequence
  - Low quality bases
- Filtering: remove bad reads from library
  - Low quality reads
  - Contaminating sequence
  - Low complexity reads (repeats)
  - Short reads
    - Short (< 20bp) reads slow down mapping software
    - Only needed if trimming was performed
- Software
  - Cutadapt
  - Galaxy, many options including cutadapt (NGS: QC and manipulation)
  - Many others: http://seqanswers.com/wiki/Software/list

Data Quality Assessment - FastQC



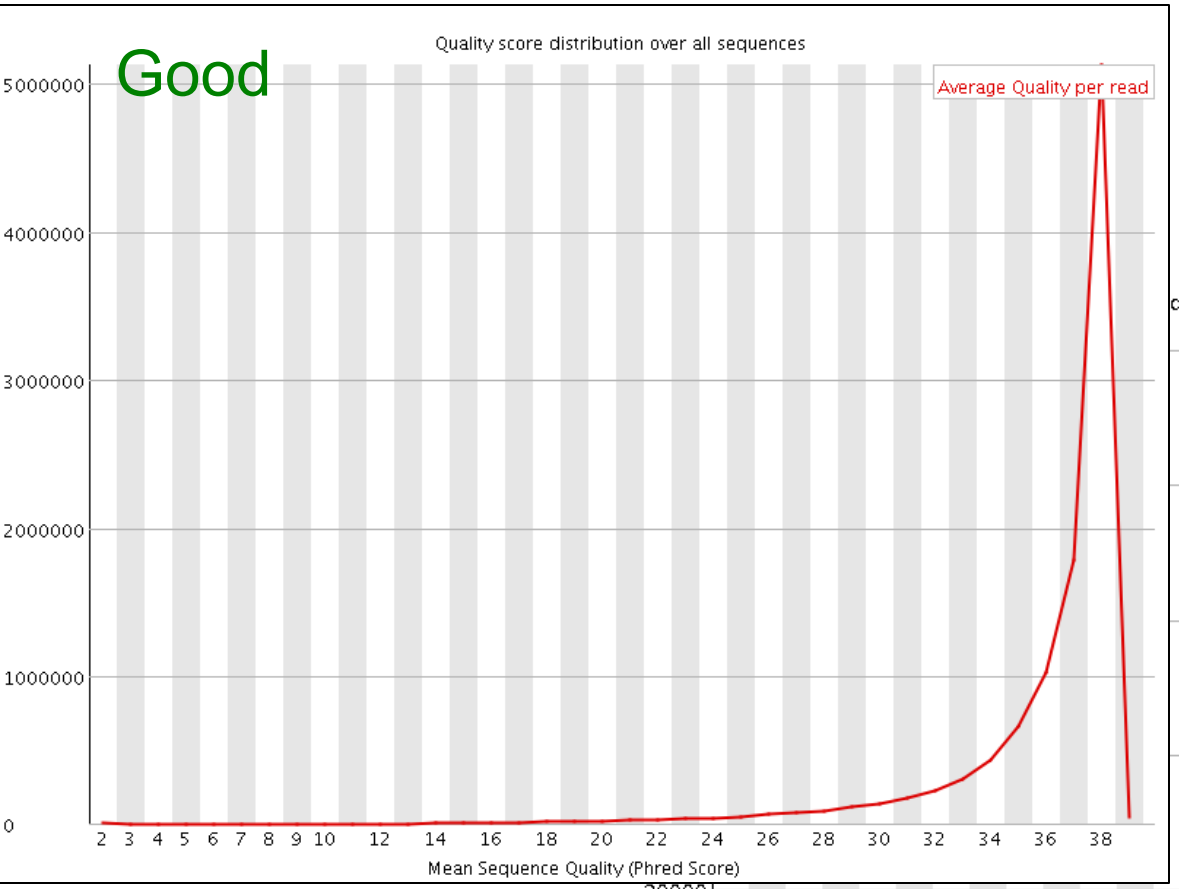
Quality scores across bases



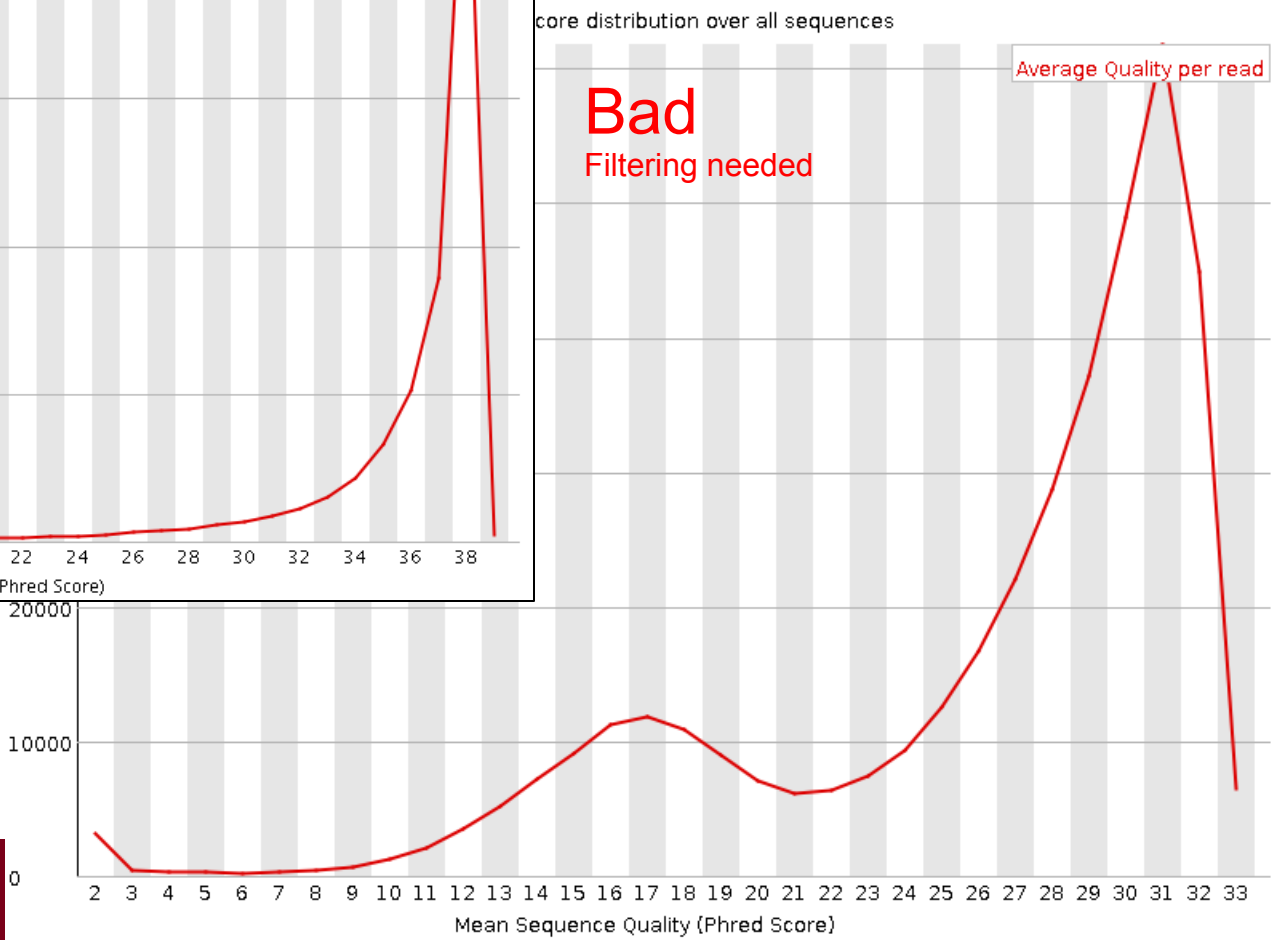
Phred 30 = 1 error / 1000 bases

Phred 20 = 1 error / 100 bases

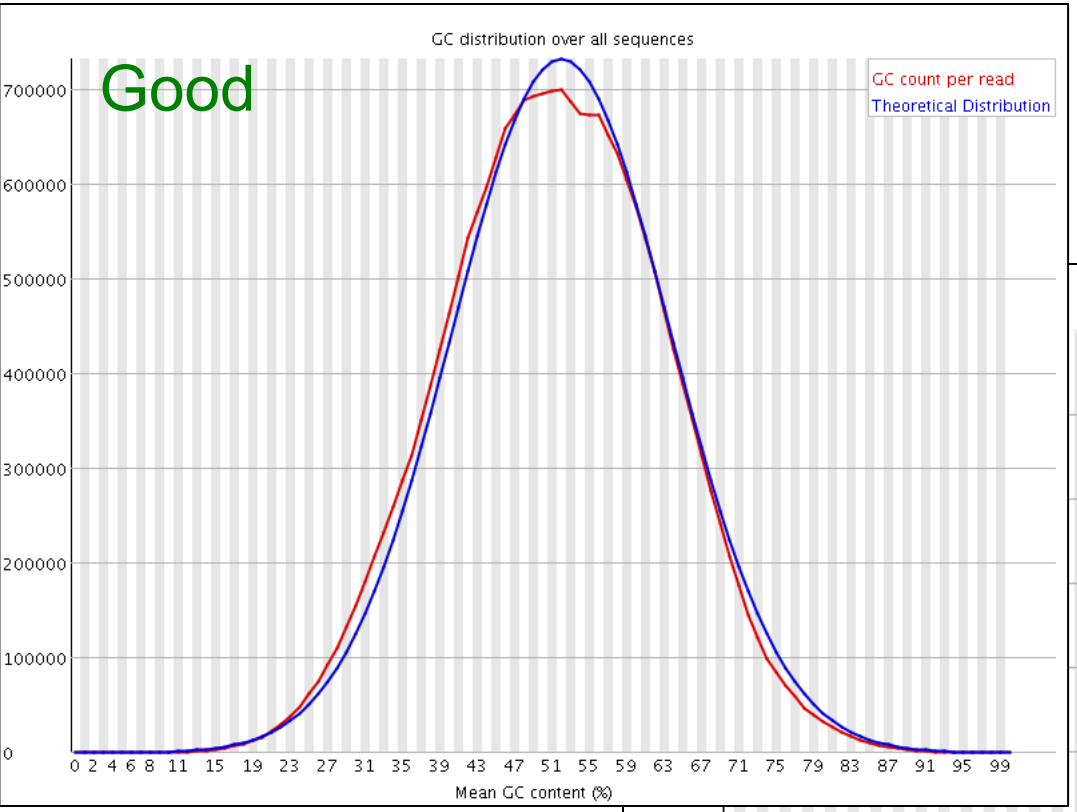
Data Quality Assessment - FastQC



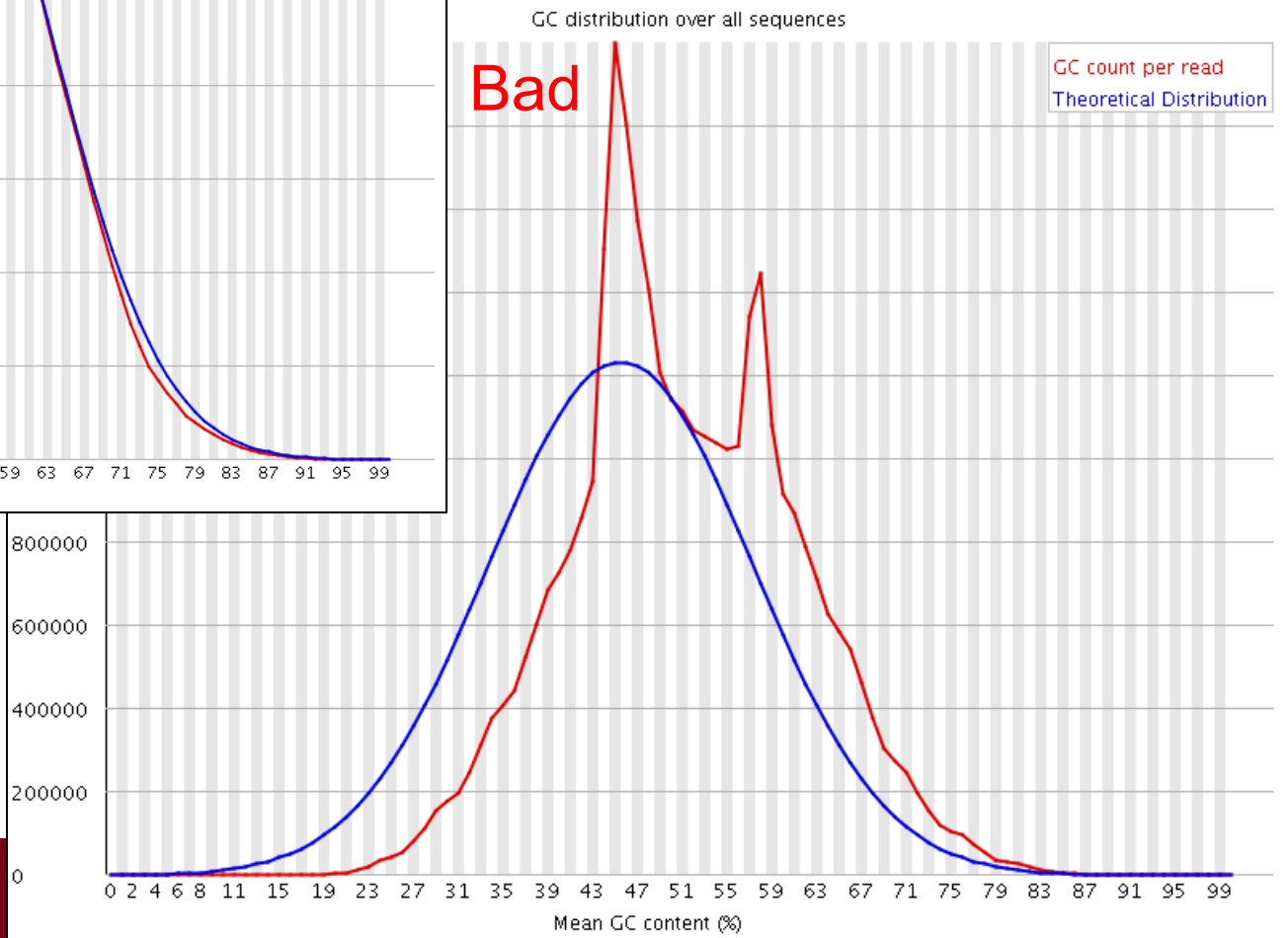
Quality scores across reads



Data Quality Assessment - FastQC



GC Distribution



Data Quality Assessment - FastQC

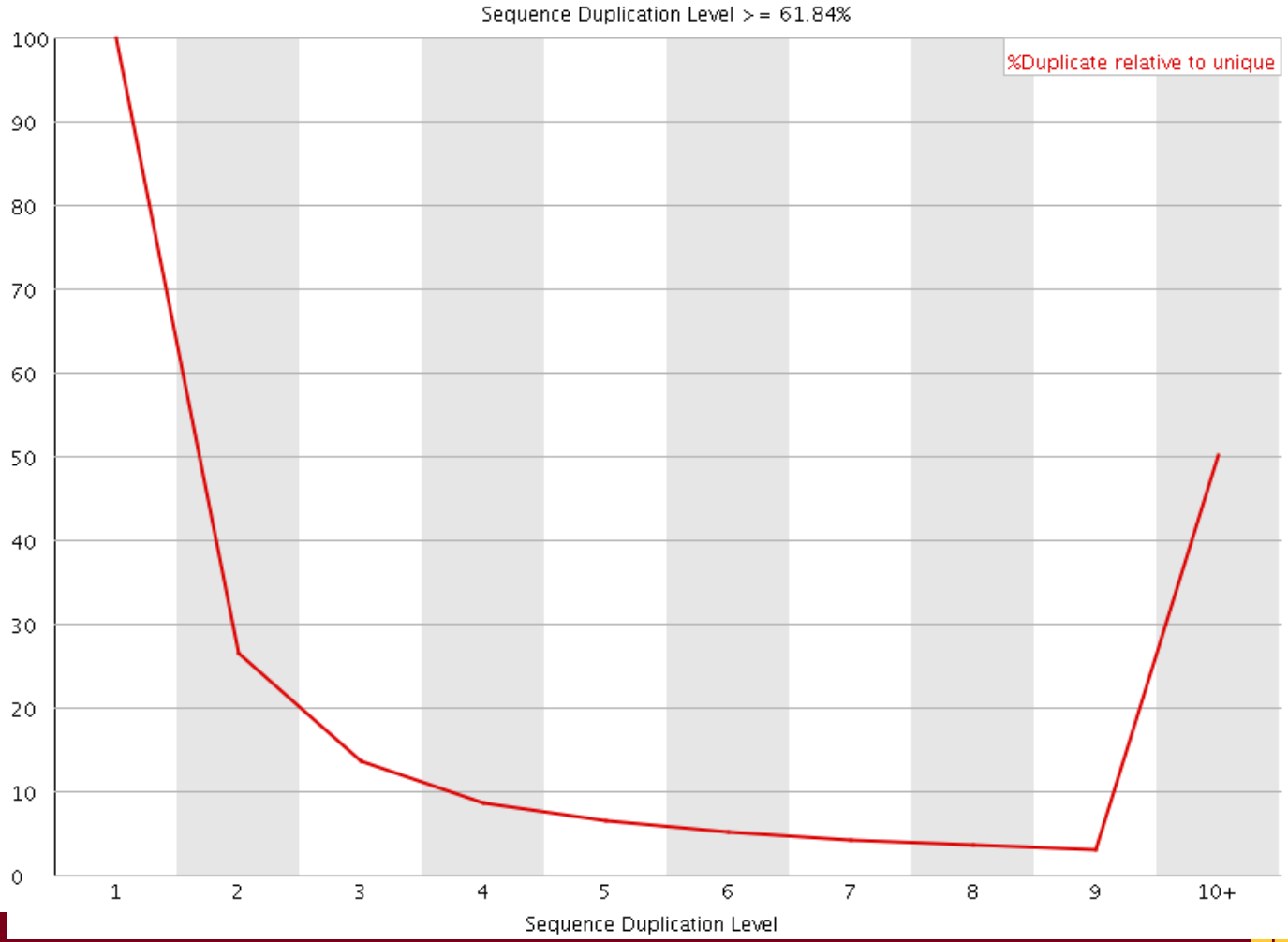
High level of sequencing adapter contamination, trimming needed

Overrepresented sequences

Sequence	Count	Percentage	Possible Source
GTATTACAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCG	820428	2.8366639370528275	Illumina Paired End PCR Primer 2 (100% over 43bp)
GTATACAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCGT	749728	2.5922157461699773	Illumina Paired End PCR Primer 2 (100% over 44bp)
CGGTTCAGCAGGAATGCCGAGATCGGAAGAGCGGTTCAGCAGGAATGCCG	648852	2.243432780066747	Illumina Paired End Adapter 2 (100% over 31bp)
GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGATCGGAAGAGCGGTTCAG	176765	0.6111723403310748	Illumina Paired End PCR Primer 2 (97% over 36bp)
ACGTCGTAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCG	143840	0.4973327832615156	Illumina Paired End PCR Primer 2 (100% over 43bp)
GTATTCAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCGT	124281	0.42970672717272257	Illumina Paired End PCR Primer 2 (100% over 44bp)
GTATCAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCGTA	99207	0.34301232917842867	Illumina Paired End PCR Primer 2 (100% over 45bp)
GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCGTATGCCGT	96289	0.33292322279941655	Illumina Paired End PCR Primer 2 (100% over 50bp)
CGGAAGAGCGGTTCAGCAGGAATGCCGAGATCGGAAGAGCGGTTCAGCAG	93842	0.3244626185124245	Illumina Paired End PCR Primer 2 (96% over 33bp)
CGTTACGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCG	75370	0.26059491013918545	Illumina Paired End PCR Primer 2 (100% over 43bp)
CGTACGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCGT	63691	0.22021428183196043	Illumina Paired End PCR Primer 2 (100% over 44bp)
ACGTAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCGTAT	56765	0.19626734873359242	Illumina Paired End PCR Primer 2 (100% over 46bp)
TACTGTAAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCG	42991	0.14864317078139472	Illumina Paired End PCR Primer 2 (100% over 43bp)

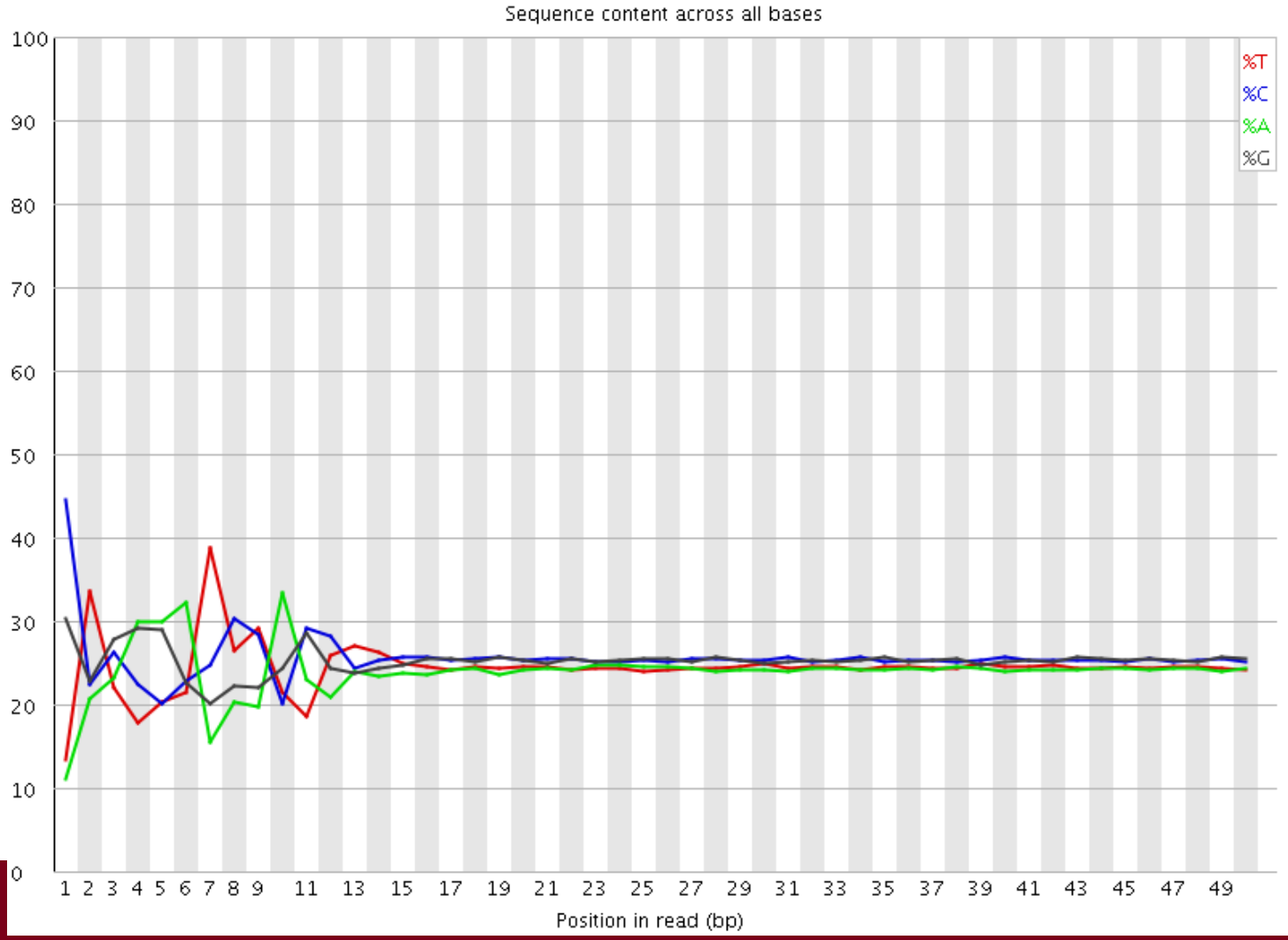
Data Quality Assessment - FastQC

Normal level of sequence duplication in 20 million read mammalian sample



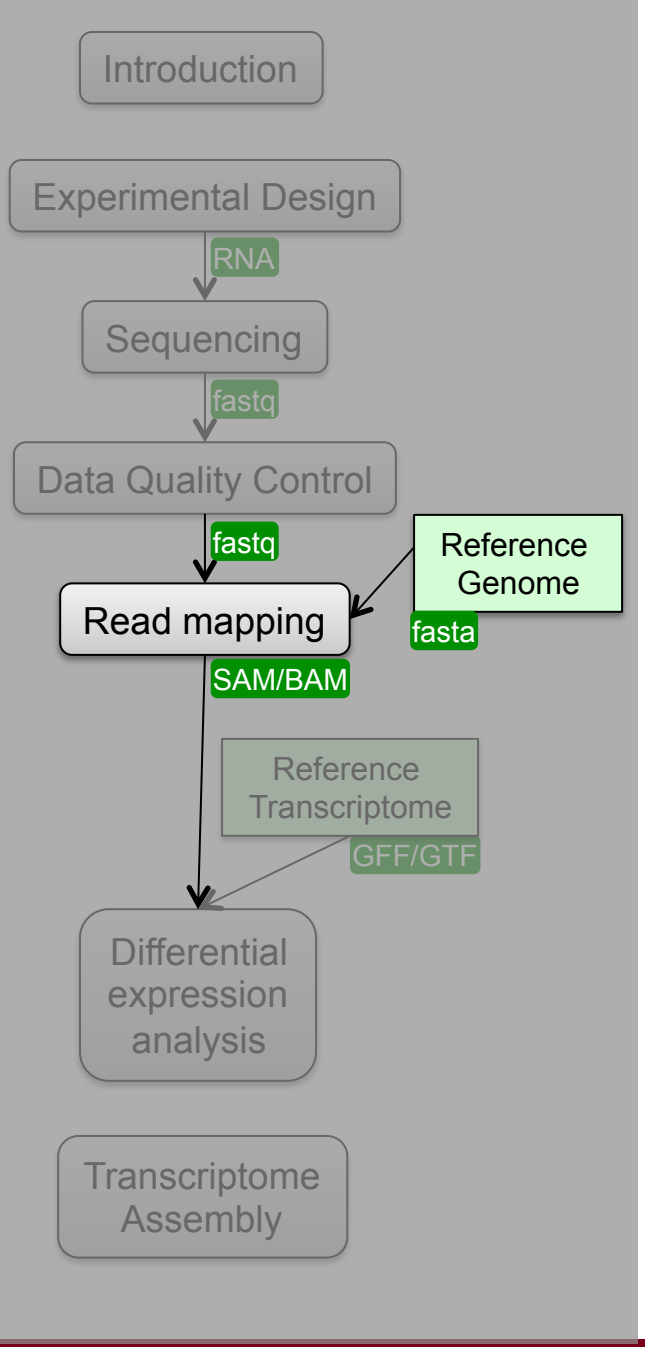
Data Quality Assessment - FastQC

Normal sequence bias at beginning of reads due to non-random hybridization of random primers



Data Quality Assessment

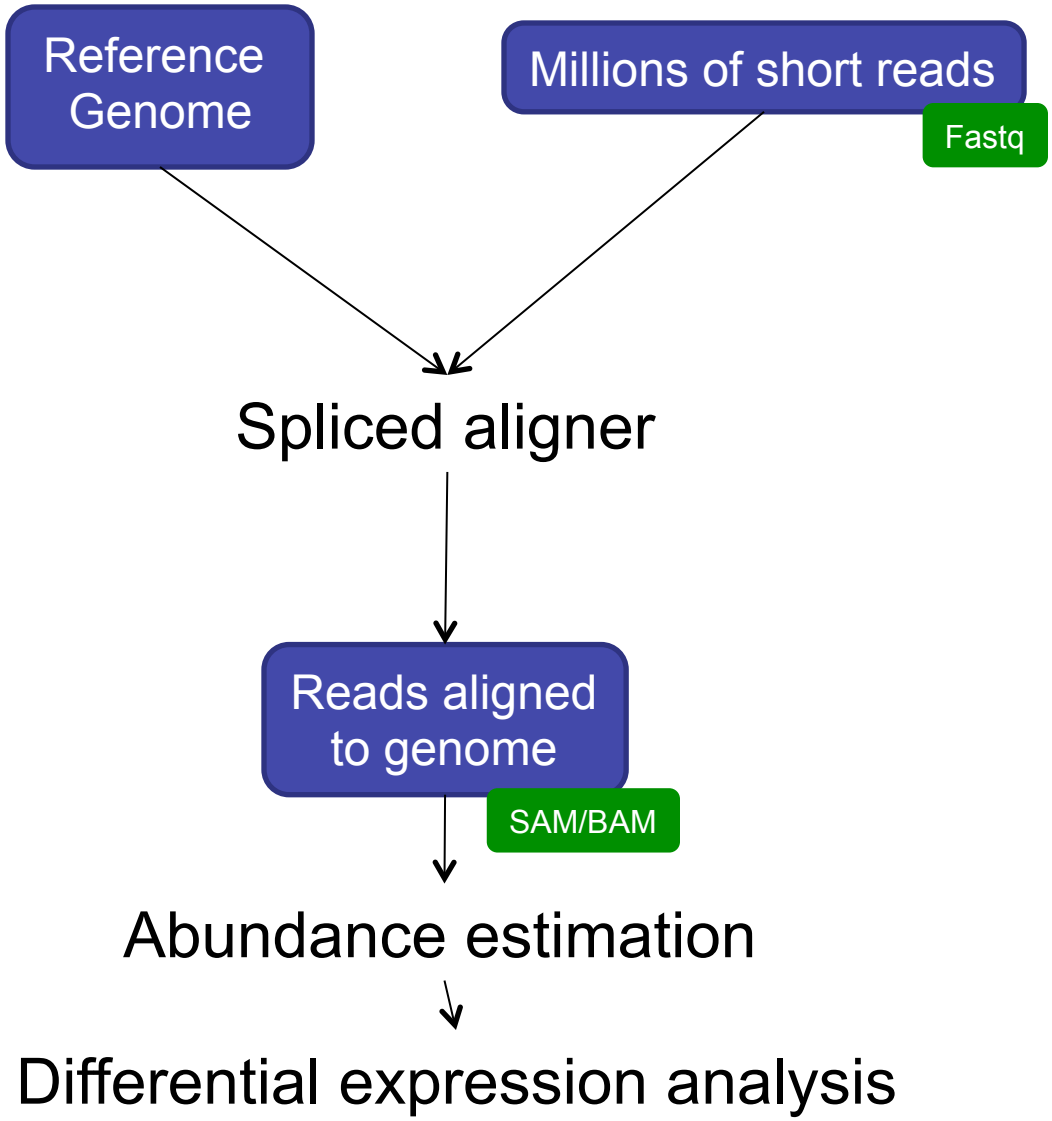
- Recommendations
  - Generate quality plots for all read libraries
  - Trim and/or filter data if needed
    - Always trim and filter for de novo transcriptome assembly
  - Regenerate quality plots after trimming and filtering to determine effectiveness



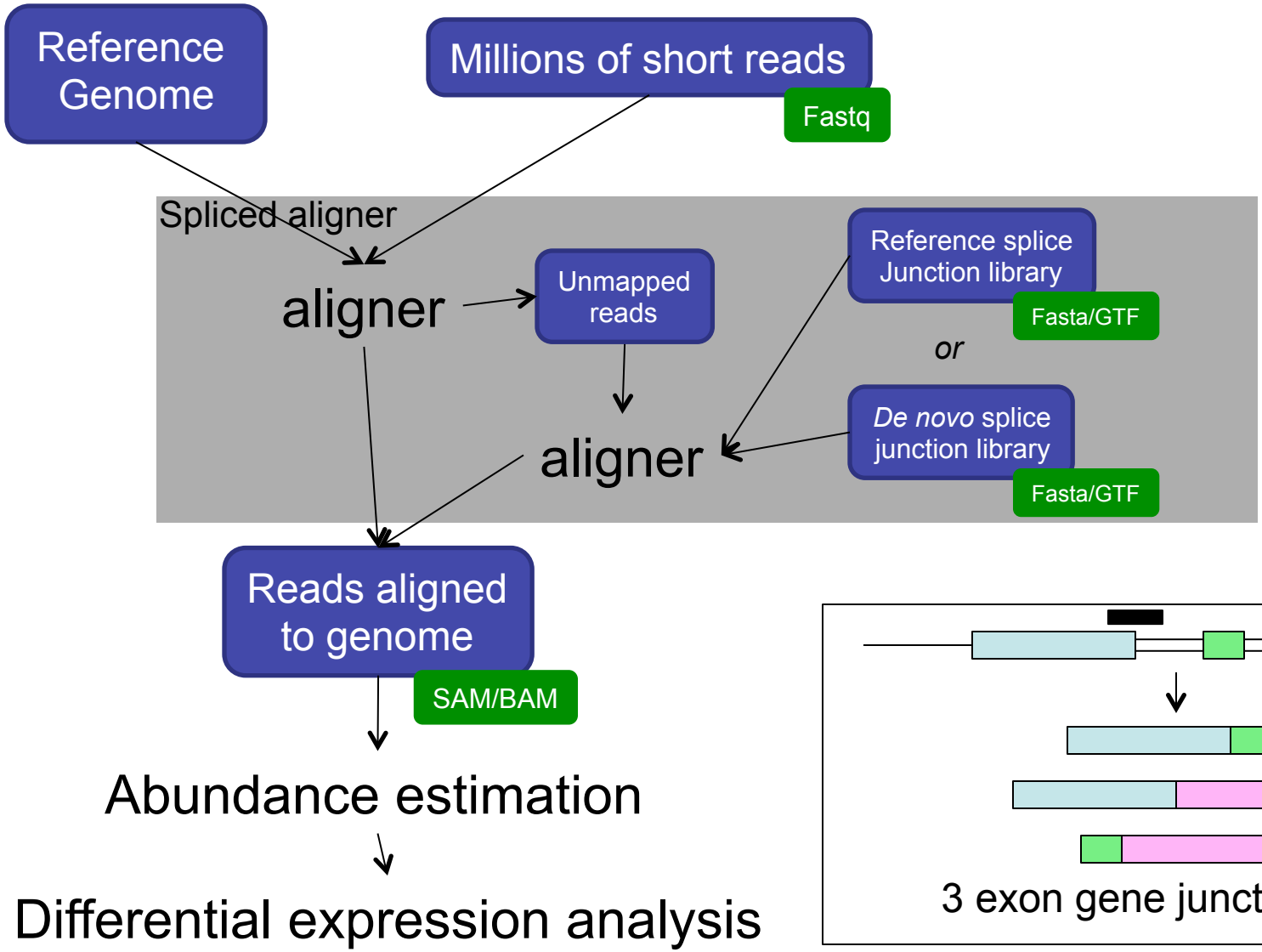
Read Mapping

- Pipeline
- Software
- Input
- Output

Mapping – with reference genome



Mapping – with reference genome

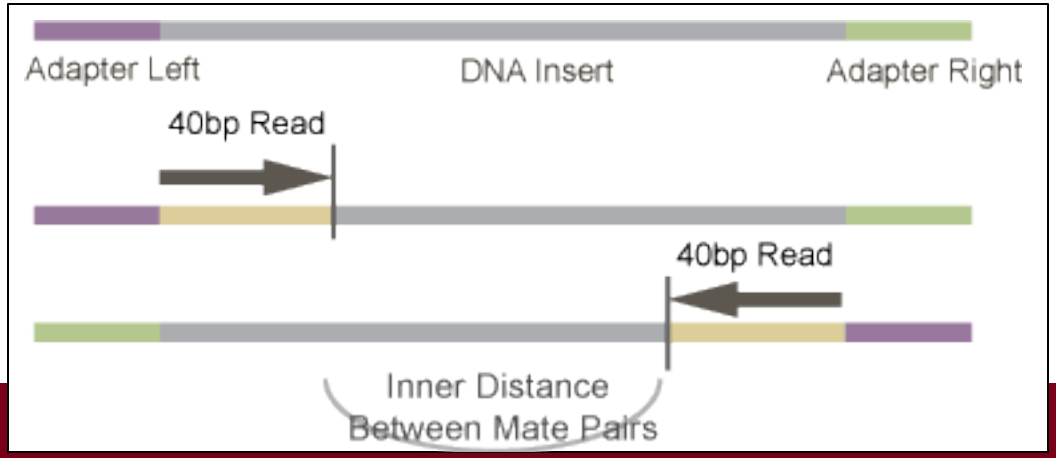


Mapping

- Alignment algorithm must be
  - Fast
  - Able to handle SNPs, indels, and sequencing errors
  - Allow for introns for reference genome alignment (spliced alignment)
- Burrows Wheeler Transform (BWT) mappers
  - Faster
  - Few mismatches allowed (< 3)
  - Limited indel detection
  - Spliced: Tophat, MapSplice
  - Unspliced: BWA, Bowtie
- Hash table mappers
  - Slower
  - More mismatches allowed
  - Indel detection
  - Spliced: GSNAP, MapSplice
  - Unspliced: SHRiMP, Stampy

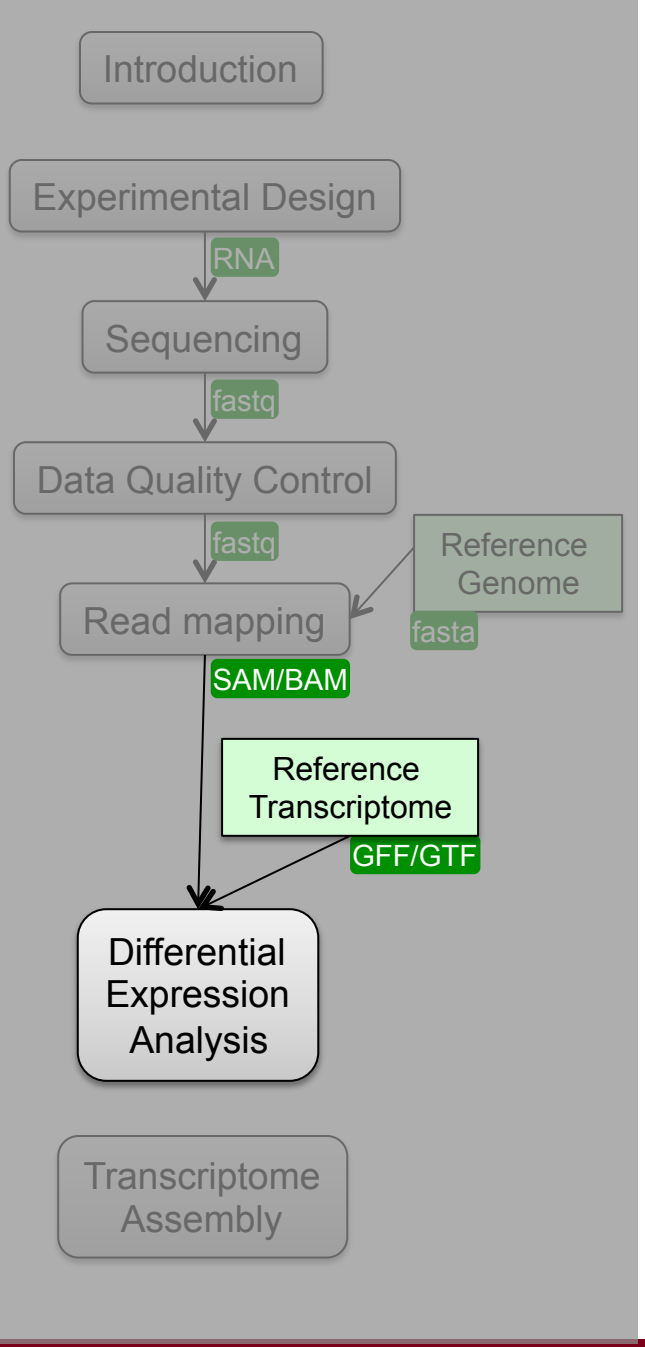
Mapping

- Input
  - Fastq read libraries
  - Reference genome index (software-specific: /project/db/genomes)
  - Insert size mean and stddev (for paired-end libraries)
    - Map library (or a subset) using estimated mean and stddev
    - Calculate empirical mean and stddev
      - Galaxy: NGS Picard: insertion size metrics
      - Cufflinks standard error
    - Re-map library using empirical mean and stddev



Mapping

- Output
  - SAM (text) / BAM (binary) alignment files
    - SAMtools – SAM/BAM file manipulation
    - Picard-tools – SAM/BAM file manipulation
  - Summary statistics (per read library)
    - % reads with unique alignment
    - % reads with multiple alignments
    - % reads with no alignment
    - % reads properly paired (for paired-end libraries)



Differential Expression

- Discrete vs continuous data
- Cuffdiff and EdgeR

Differential Expression

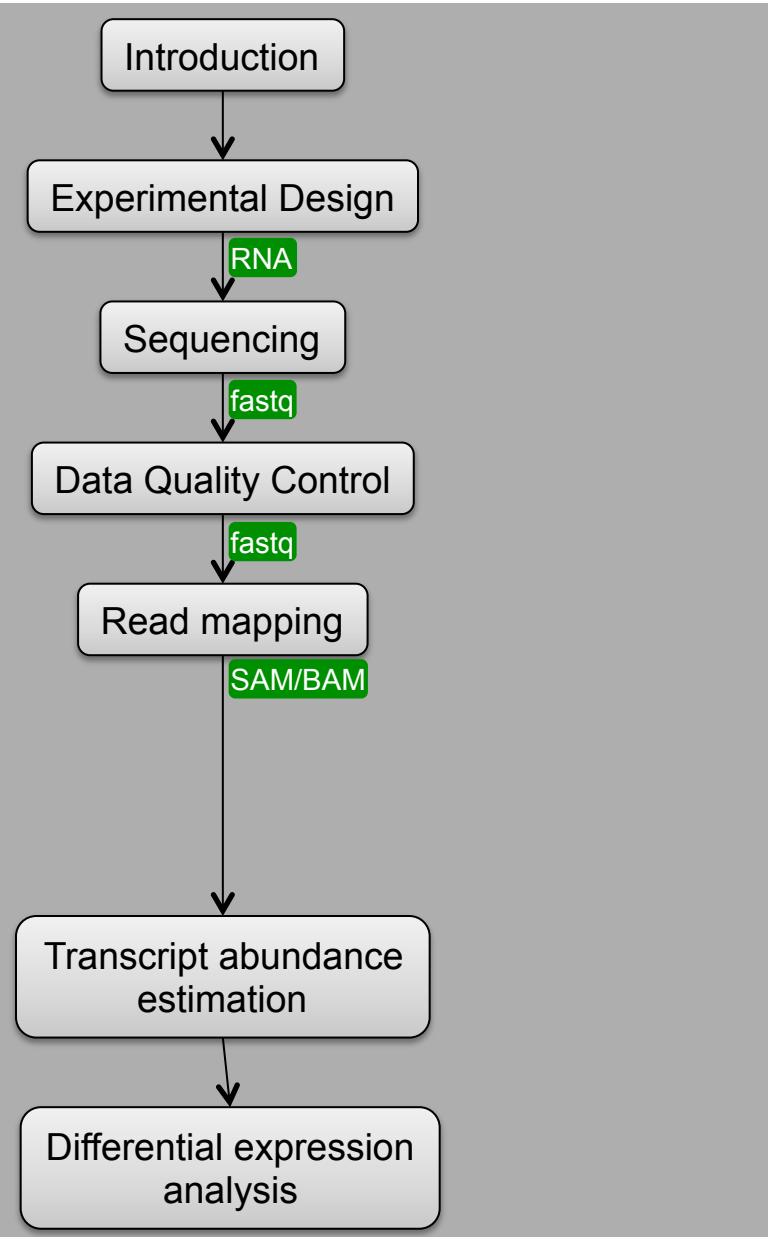
- Discrete vs Continuous data
  - Microarray florescence intensity data: continuous
    - Modeled using normal distribution
  - RNA-Seq read count data: discrete
    - Modeled using negative binomial distribution

Microarray software cannot be used to analyze RNA-Seq data

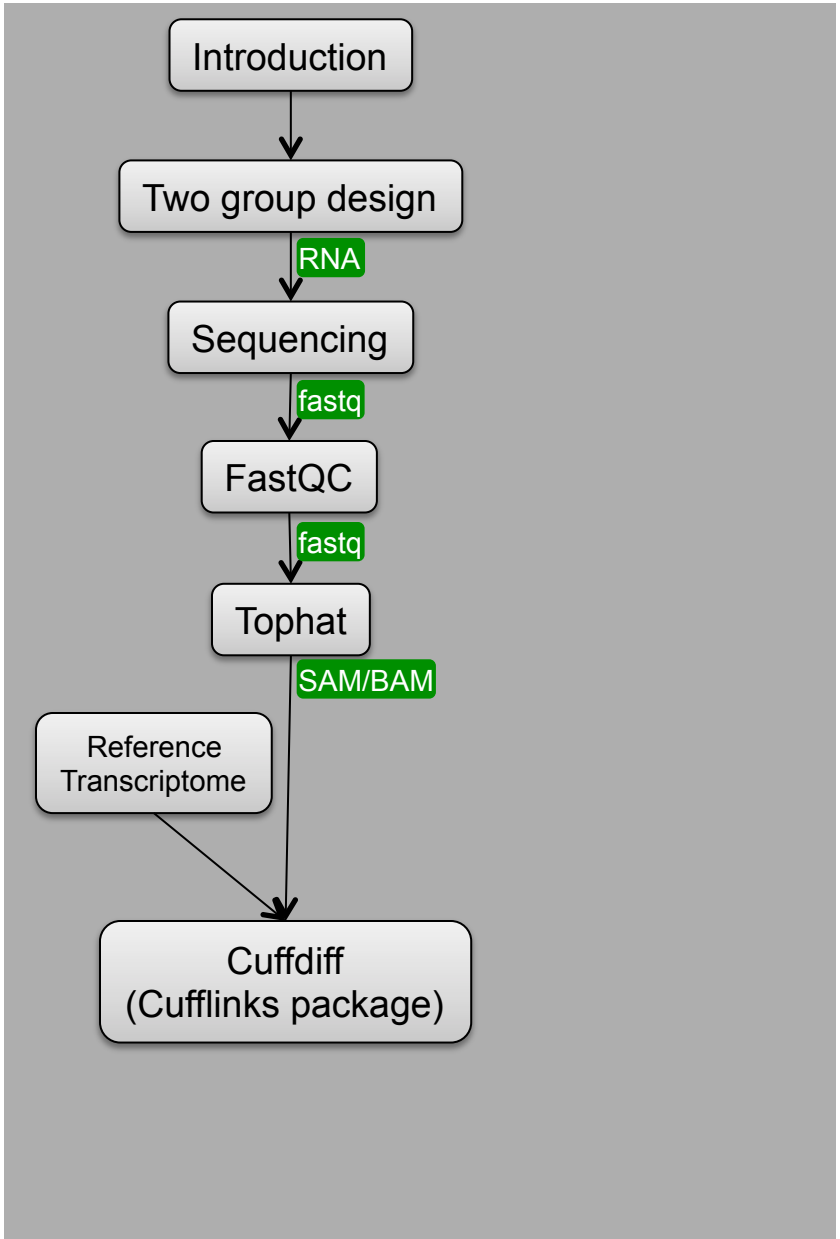
Differential Expression

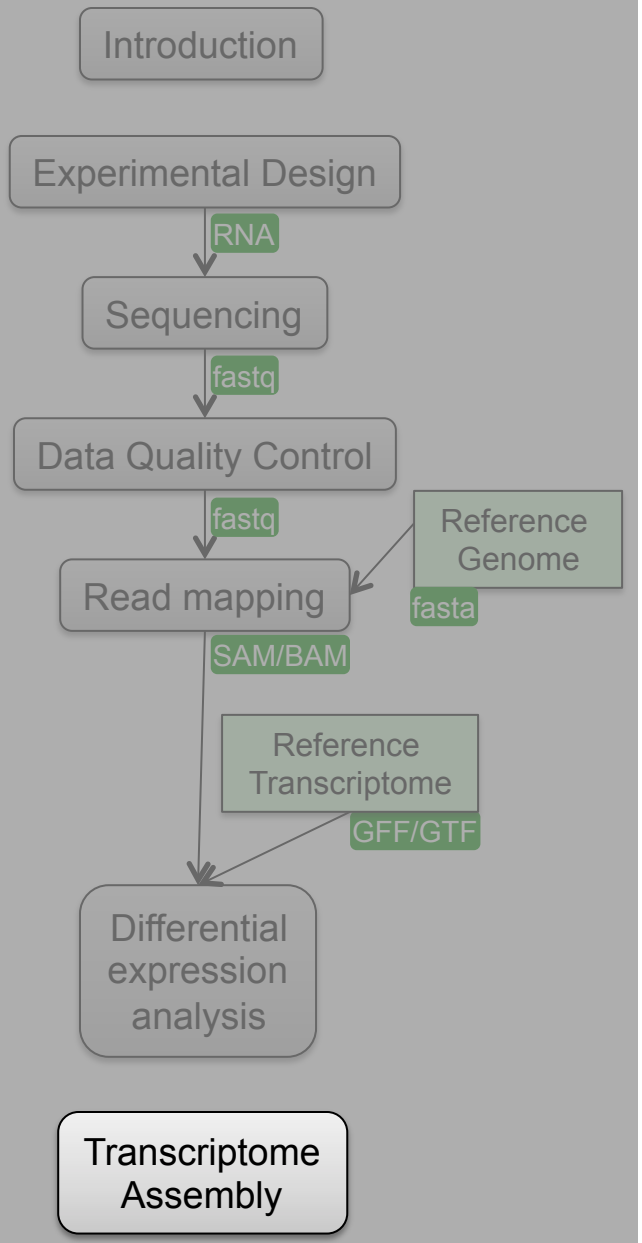
- Cuffdiff (Cufflinks package)
  - Pairwise comparisons
  - Differential gene, transcript, and primary transcript expression; differential splicing and promoter use
  - Easy to use, well documented
  - Input: transcriptome, SAM/BAM read alignments (abundance estimation built-in)
- EdgeR
  - Complex experimental designs using generalized linear model
  - Information sharing among genes (Bayesian gene-wise dispersion estimation)
  - Difficult to use R package — 

  - Input: raw gene/transcript read counts (calculate abundance with separate software *not cufflinks*)
- Others
  - DESeq - R package
  - edgeR – R package
  - RSEM – abundance estimation
  - HTSeq – abundance estimation



Suggested Implementation
(reference genome available)

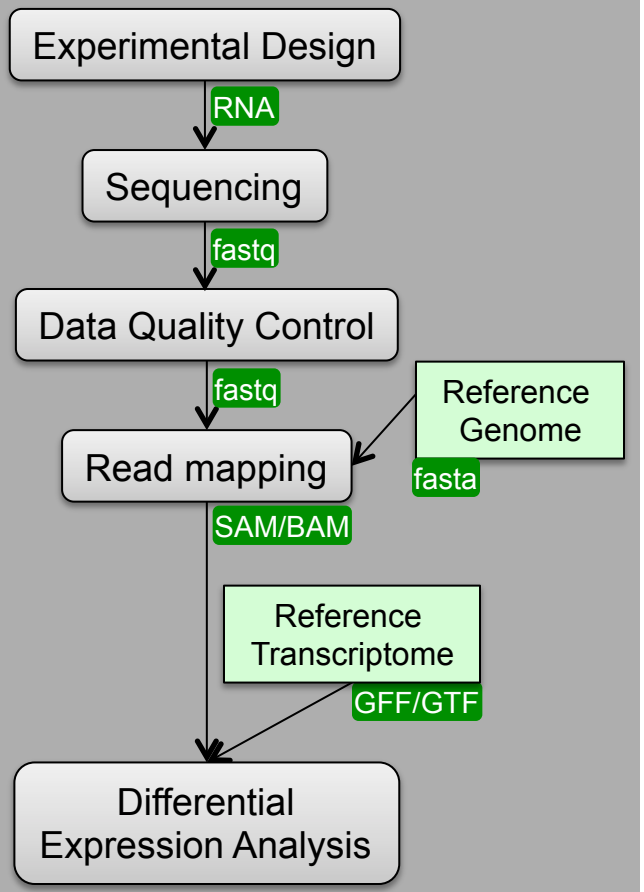




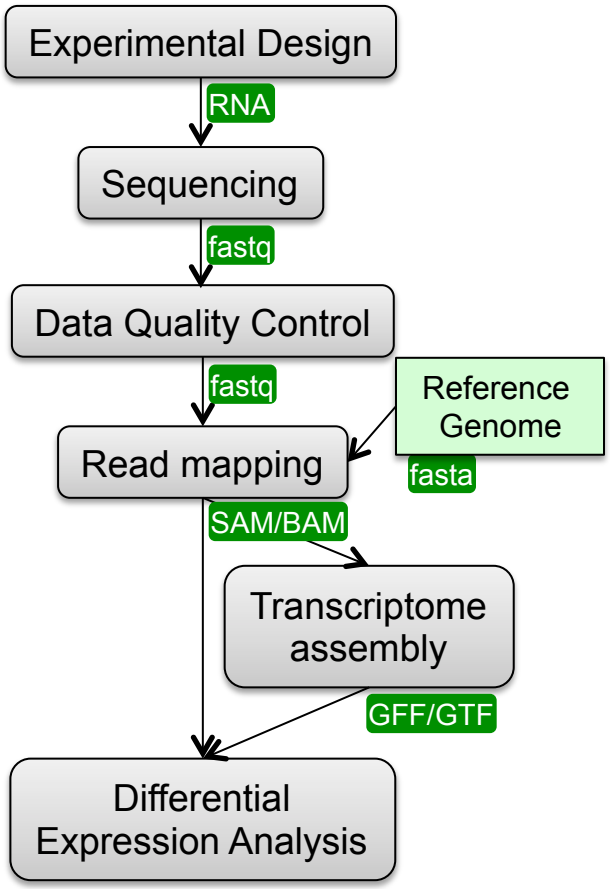
Transcriptome Assembly

- Pipeline
- Software
- Input
- Output

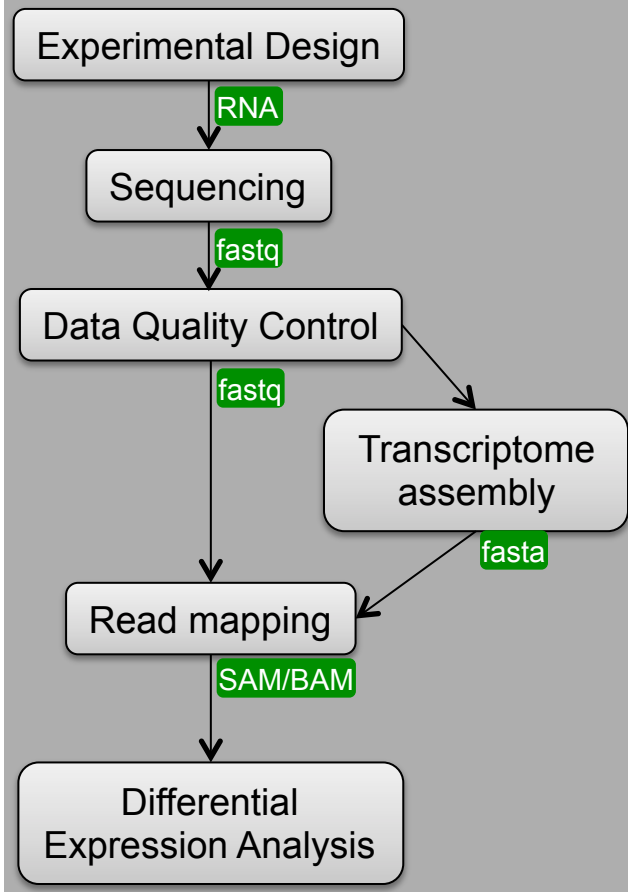
RNA-Seq
- Reference genome
- Reference transcriptome



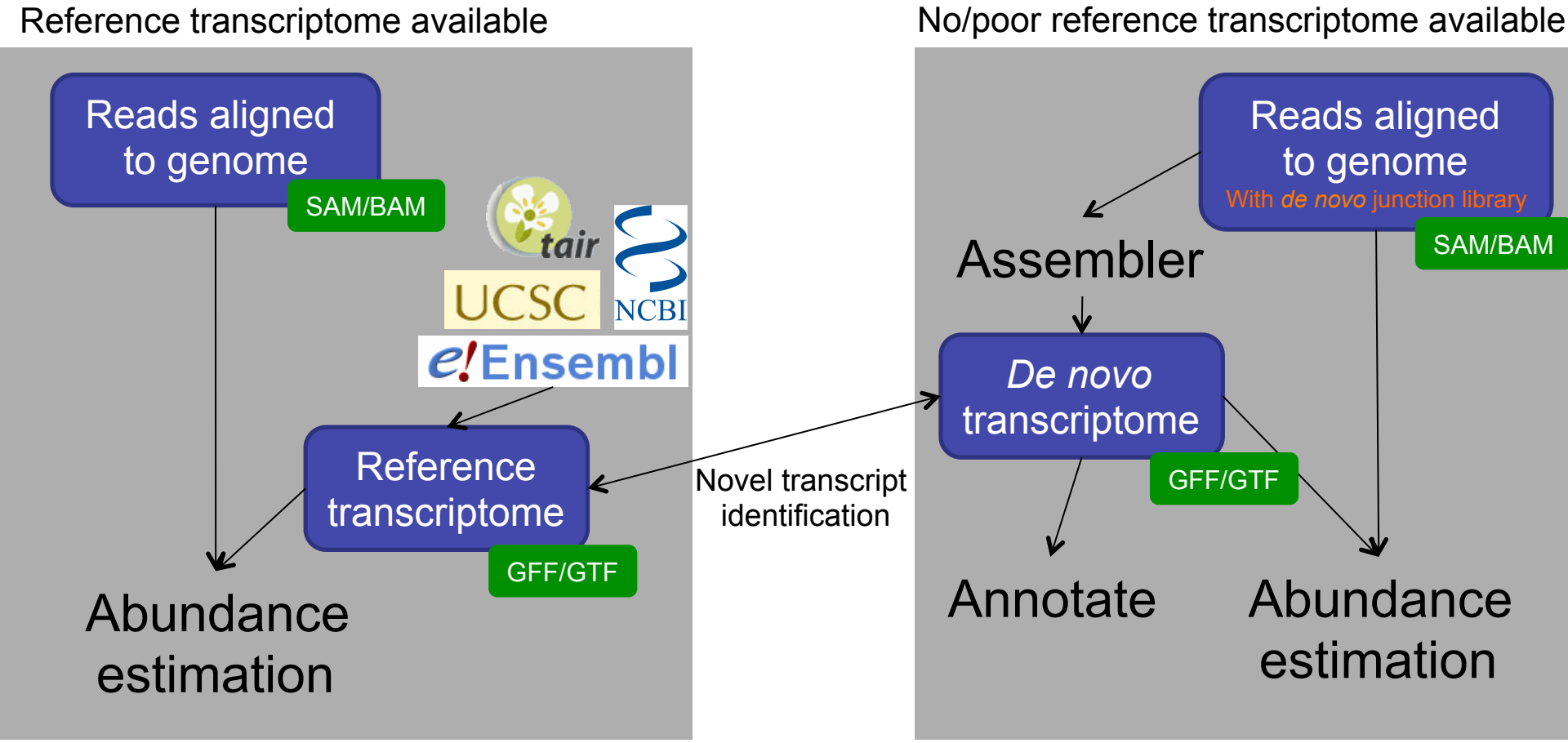
RNA-Seq
- Reference genome
- No reference transcriptome



RNA-Seq
- No reference genome
- No reference transcriptome



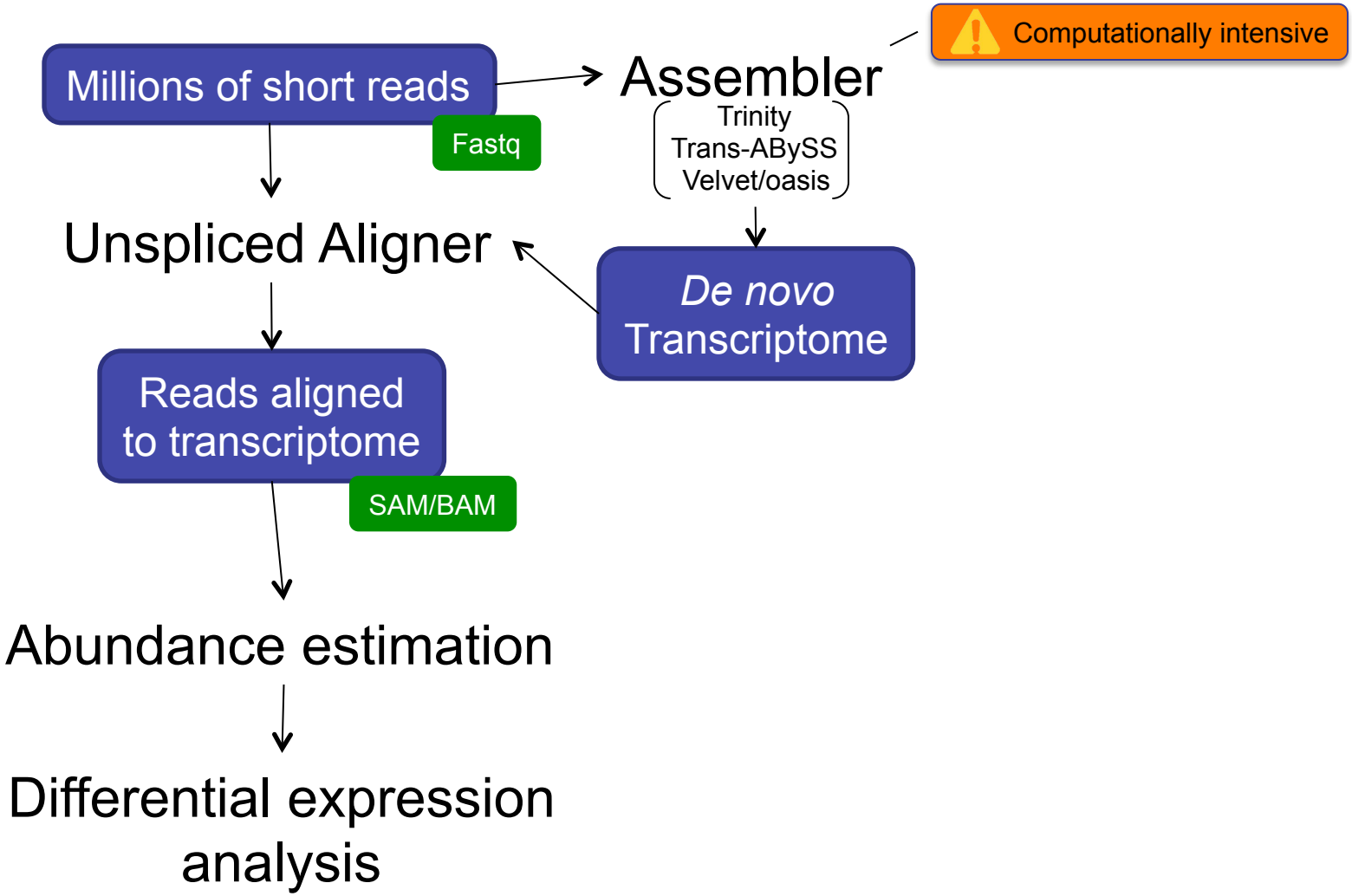
Transcriptome Assembly -with reference genome



Transcriptome Assembly -with reference genome

- Reference genome based assembly
  - Cufflinks, Scripture
- Reference annotation based assembly
  - Cufflinks
- Transcriptome comparison
  - Cuffcompare
- Transcriptome Annotation
  - Generate cDNA fasta from annotation (Cufflinks' gffread program)
  - Align to library of known cDNA (RefSeq, GenBank)

Transcriptome Assembly – no reference genome



Further Reading

Bioinformatics for High Throughput Sequencing

Online access through U library

Rodríguez-Ezpeleta, Naiara.; Hackenberg, Michael.; Aransay, Ana M.;
SpringerLink New York, NY : Springer c2012

RNA sequencing: advances, challenges and opportunities

Fatih Ozsolak1 & Patrice M. Milos1
Nature Reviews Genetics 12, 87-98 (February 2011)

Computational methods for transcriptome annotation and quantification using RNA-seq

Manuel Garber, Manfred G Grabherr, Mitchell Guttman & Cole Trapnell
Nature Methods 8, 469–477 (2011)

Table of RNA-Seq software

Next-generation transcriptome assembly

Jeffrey A. Martin & Zhong Wang
Nature Reviews Genetics 12, 671-682 (October 2011)

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 Kelley, Harold Pimentel, Steven Salzberg, John L Rinn & Lior Pachter
Nature Protocols 7, 562–578 (2012)

SEQanswers.com

biostar.stackexchange.com

Popular bioinformatics forums

Questions / Discussion