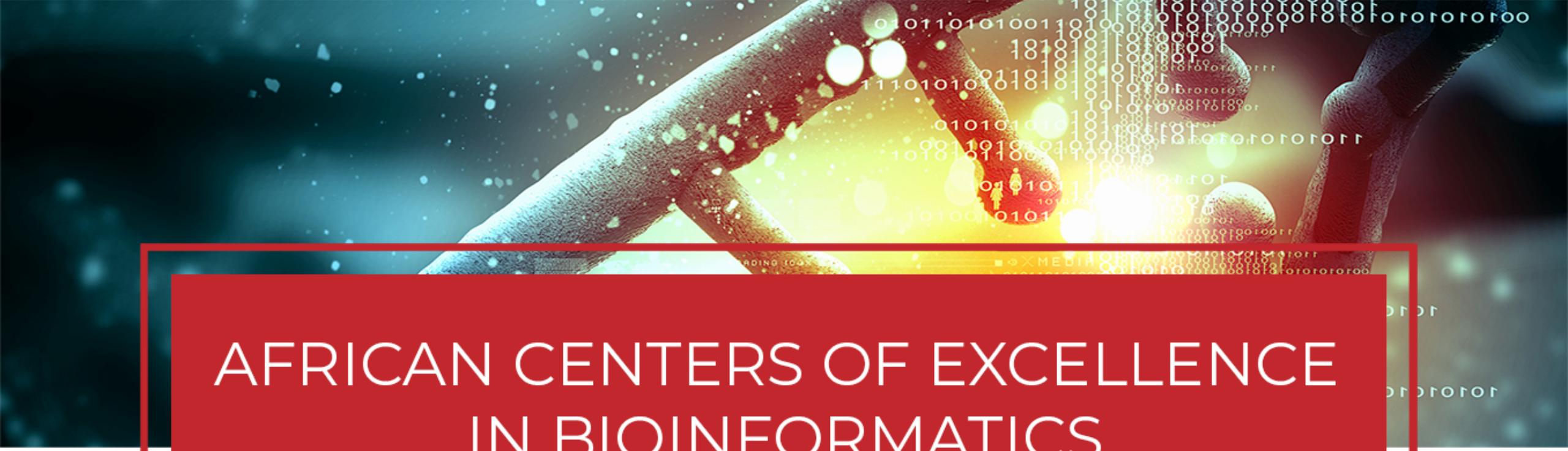

AFRICAN CENTERS OF EXCELLENCE IN BIOINFORMATICS

KAMPALA, UGANDA

Processing and Analysis of RNAseq NGS Data

Today's Instructor

Brendan Jeffrey, Ph.D. Molecular and Cellular Biology

- Bioinformatics and Computational Biosciences Branch (BCBB), NIAID
- National Institutes of Health, Bethesda, MD USA
- NIH - Rocky Mountain Labs, Hamilton, MT USA
- Contact our team via email:
  - Listserv: bioinformatics@niaid.nih.gov
  - Instructor: brendan.jeffrey@nih.gov

Purpose of This Course

National Institute of Allergy and Infectious Diseases

Introduction to RNAseq

An introduction to computational tools used for processing and analyzing RNAseq data – heavy on the R

Course materials - https://github.com/niaid/ACE_Uganda_2021_RNAseq

Introduction – What is RNAseq

National Institute of Allergy and Infectious Diseases

RNA sequencing uses next-generation sequencing to reveal presence and quantity of RNA in a biological sample at a given moment in time

Introduction – Why Sequence RNA

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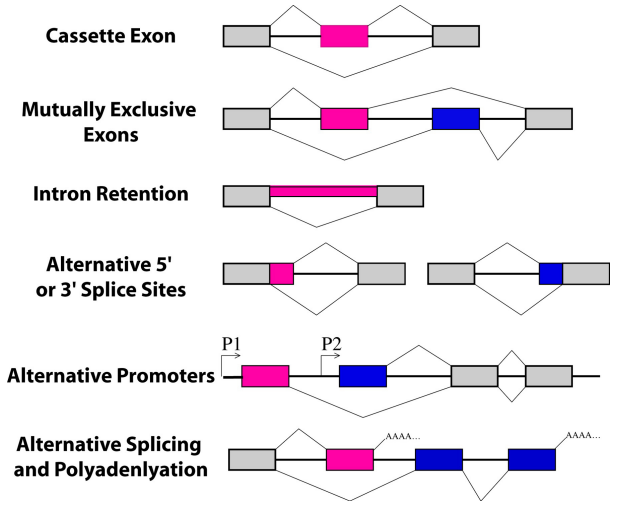
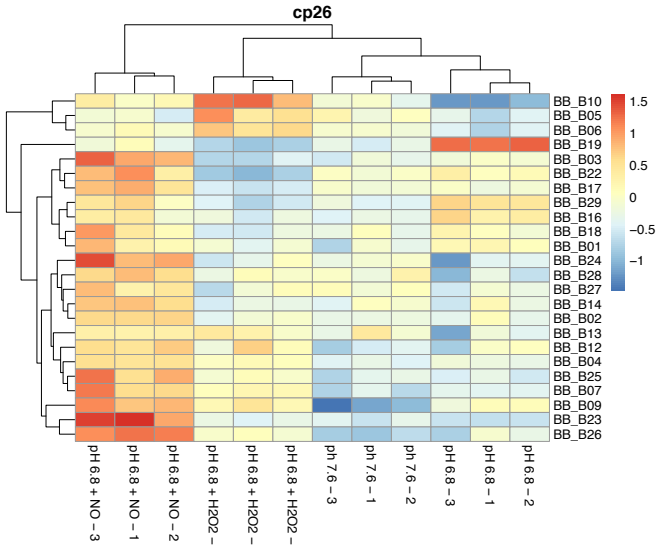
Qualitative - Annotation

- Transcriptome assembly
- Novel gene finding
- Defining Exon/Intron boundaries
- SNP finding



Quantitative

- Functional studies
- Differential gene expression
- Alternative splicing



RNAseq – Experiment Workflow

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Design Experiment

Experiment designed to address your question

RNA Preparation

Isolate and purify RNA

Prepare Libraries

Convert RNA to cDNA add adaptors, multiplex barcodes

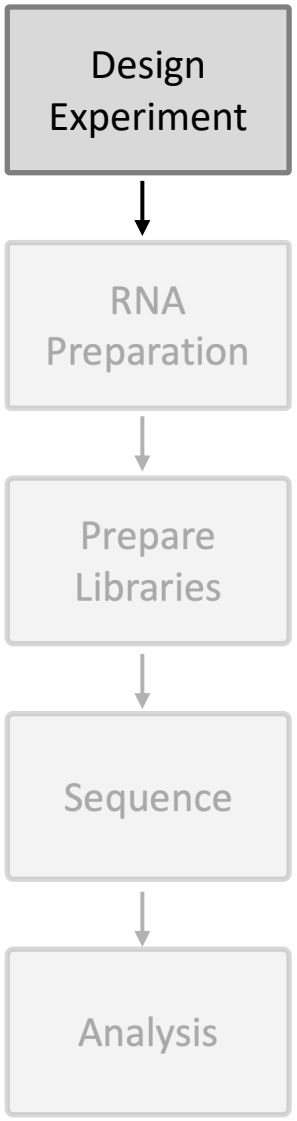
Sequence

Sequence cDNA using sequencing platform - Illumina / Pac Bio

Analysis

Analyzing sequenced short reads

RNAseq – Experiment Workflow



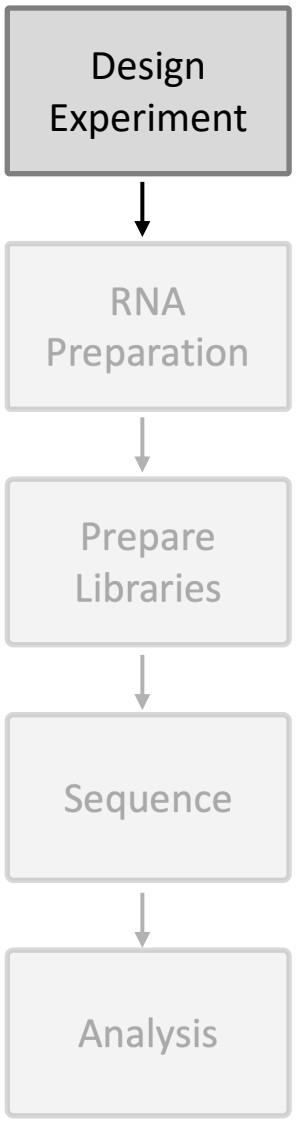
Qualitative/Annotation – analyze RNAseq reads to identify genes, architecture

Depth of coverage is important versus replicates

Library prep methods important to get even coverage across entire transcript

SMARTer system - clontech

RNAseq – Experiment Workflow



Differential Gene Expression

DGE experiments designed to accurately measure both the counts of each gene (coverage) and the variances that are associated with those counts (replicates)

Biological replicates >> Technical replicates

Biometry: The Principles and Practices of Statistics in Biological Research – Sokal and Rohlf

RNAseq – Experiment Workflow

National Institute of Allergy and Infectious Diseases

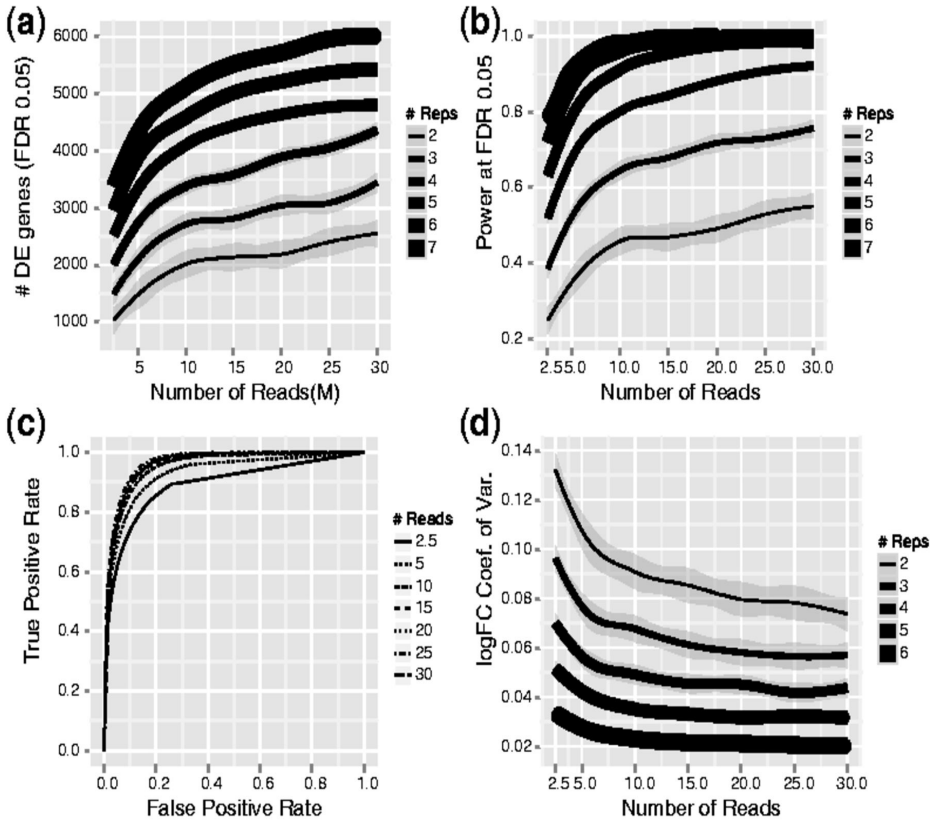
Design Experiment

RNA Preparation

Prepare Libraries

Sequence

Analysis



Drug 1 with 2 treatments (+ or -)

+ N = 6	- N = 6

Total # ind. = 12

Drug 1 with 2 treatments (+ or -) by Drug 2 with 2 treatments (+ or -) by Drug 3 with 2 treatments (+ or -)

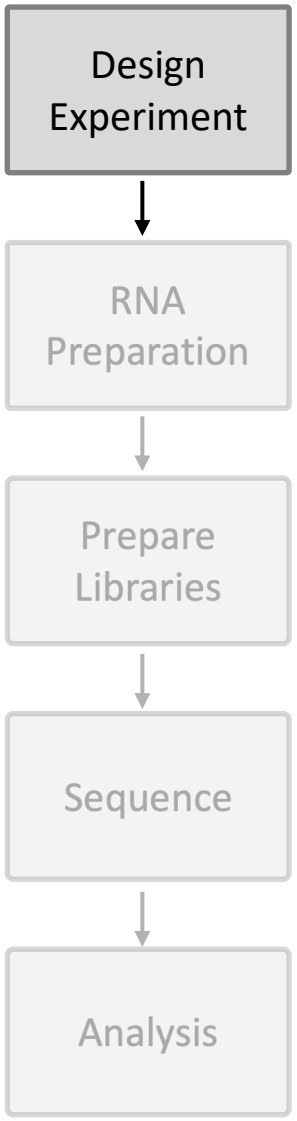
- / - / - N = 6	- / - / + N = 6	- / + / - N = 6	- / + / + N = 6
+ / - / - N = 6	+ / - / + N = 6	+ / + / - N = 6	+ / + / + N = 6

Total # ind. = 48

Sample treatment/processing randomization

RNAseq – Experiment Workflow

National Institute of Allergy and Infectious Diseases



We need more power! - Scotty

Inputs

Pilot Data: Upload your own pilot data or used a stored dataset as a model for your experiment. (?)

CAUTION
Power analysis results will not be predictive of the actual results unless the power analysis is performed on data that closely matches the experiment. Please read about generating pilot data and selecting preloaded datasets before continuing.

Upload Data

Upload a file containing the number of reads per gene for pilot data as a tab delimitted text file. See format info.

Choose File No file chosen

Number of Replicates in Control: 2

Number of Replicates in Test (enter 0 if none): 2

Use a stored dataset(?)

Choose a model dataset *(Less Accurate)*: I have my own pilot data. Dataset Descriptions

Cost Data (?)

Cost per replicate, excluding reads:

Control:

Test:

Cost per million reads sequenced: (?)

Alignment Rate (to genes or transcripts): 50 % (How to calculate?)

Constraints for Power Optimization(?)

Experimental Configurations to Test:

Maximum number of biological replicates per condition: 10

Assess the power of sequencing depths between 10,000,000 and 100,000,000 reads aligned to genes per replicate

RNAseq – Experiment Workflow

National Institute of Allergy and Infectious Diseases

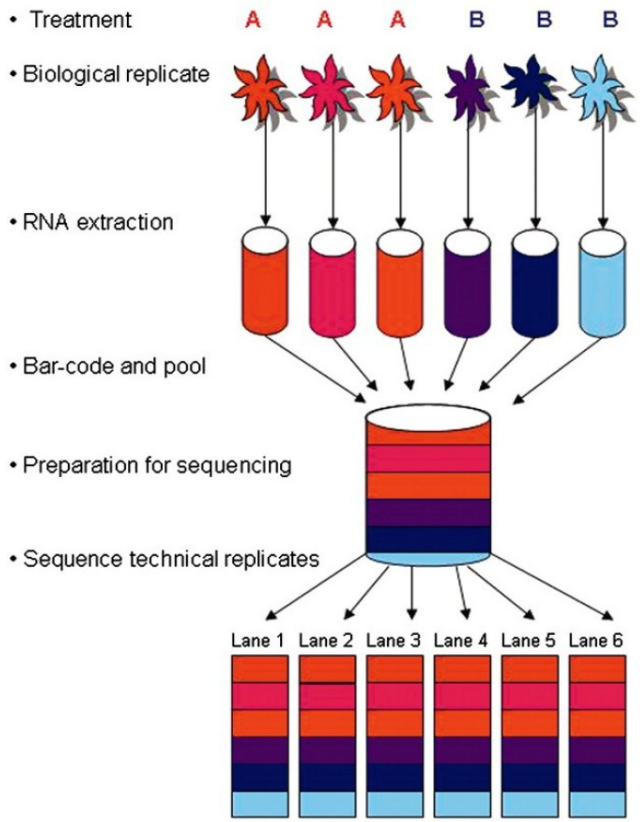
Design Experiment

RNA Preparation

Prepare Libraries

Sequence

Analysis



Multiplex and pool when sequencing

Auer P L , and Doerge R W Genetics 2010;185:405-416

Balanced Blocked Design

RNAseq – Sequence Quality Control

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Design Experiment

RNA Preparation

Prepare Libraries

Sequence

Analysis

Several million reads generated - Fastq format

```
@M03264:1:000000000-AB9MU:1:1101:9844:1771 1:N:0
TATTCTGTGACTAGATTTTTTGAGAATAAATCAGAATACAATTCATTCTTTGATATACAAGTATCGCCCAT
+
CGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGFC<FCFFDGGGGGGGGGGGCGFGGGFFFGGGGGGGGGG
```

Table 1 ASCII Characters Encoding Q-scores 0–40

Symbol	ASCII Code	Q-Score	Symbol	ASCII Code	Q-Score	Symbol	ASCII Code	Q-Score
!	33	0	/	47	14	=	61	28
"	34	1	0	48	15	>	62	29
#	35	2	1	49	16	?	63	30
$	36	3	2	50	17	@	64	31
%	37	4	3	51	18	A	65	32
&	38	5	4	52	19	B	66	33
'	39	6	5	53	20	C	67	34
(	40	7	6	54	21	D	68	35
)	41	8	7	55	22	E	69	36
*	42	9	8	56	23	F	70	37
+	43	10	9	57	24	G	71	38
,	44	11	:	58	25	H	72	39
-	45	12	;	59	26	I	73	40
.	46	13	<	60	27			

RNAseq – Sequence Quality Control

National Institute of Allergy and Infectious Diseases

Design Experiment

RNA Preparation

Prepare Libraries

Sequence

Analysis

Several million reads generated - Fastq format

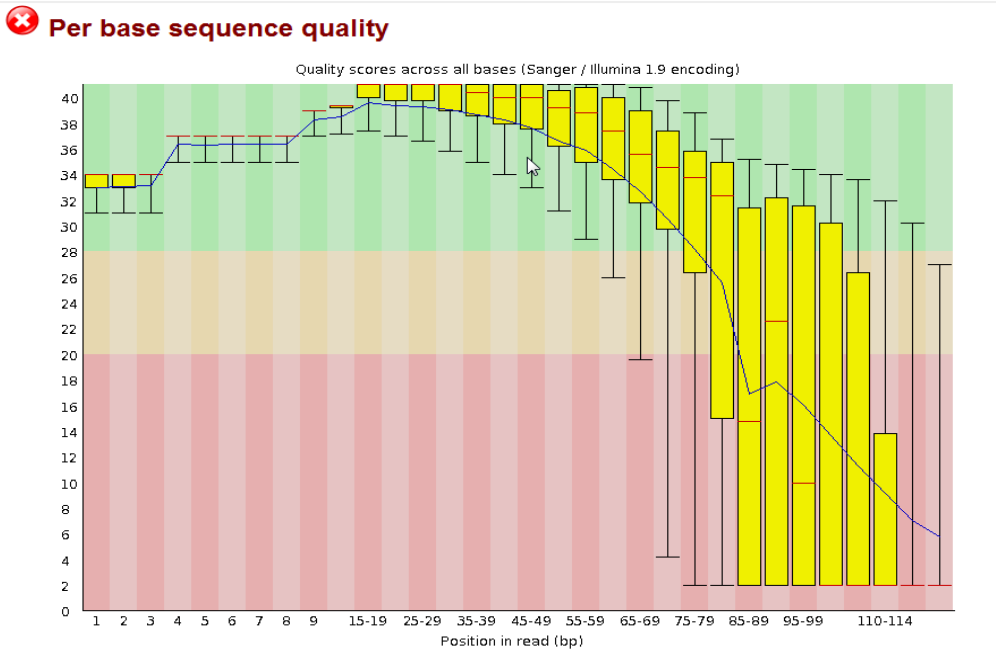
```
@M03264:1:000000000-AB9MU:1:1101:9844:1771 1:N:0
TATTCTGTGACTAGATTTTTTGAGAATAAATCAGAATACAATTCATTCTTTGATATACAAGTATCGCCCAT
+
CGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGFC<FCFFDGGGGGGGGGGGCGFGGGFFFGGGGGGGGGG
```

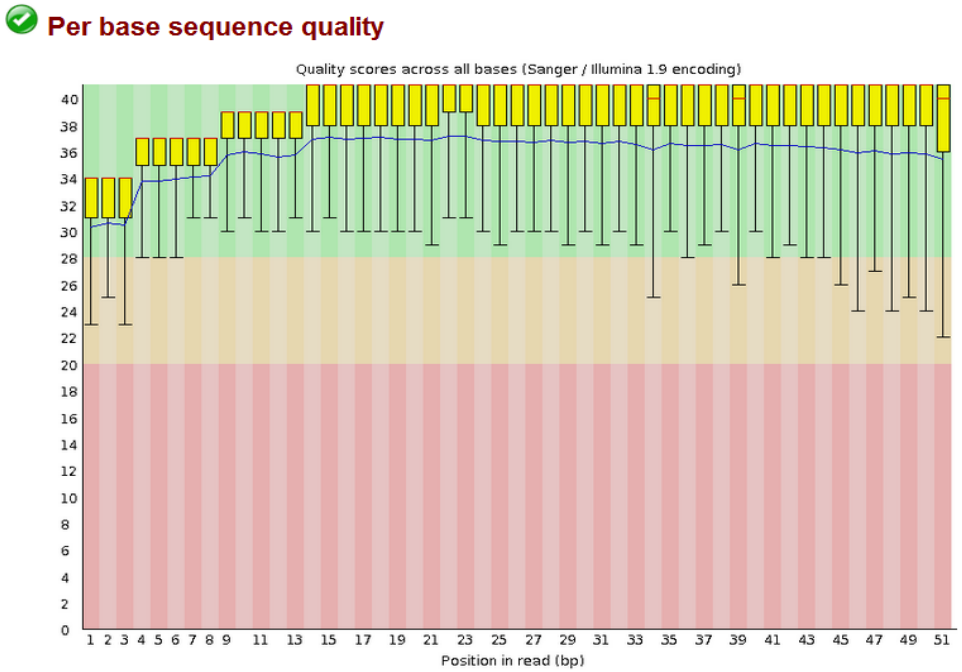
Quality Score	**Probability of Incorrect Base Call**	**Inferred Base Call Accuracy**
10 (Q10)	1 in 10	90%
20 (Q20)	1 in 100	99%
30 (Q30)	1 in 1000	99.9%

RNAseq – Sequence Quality Control

National Institute of Allergy and Infectious Diseases

- Design Experiment
- RNA Preparation
- Prepare Libraries
- Sequence
- Analysis





Read QC overview with FastQC

Illumina adaptor removal, quality trimming of reads

Trimmomatic, Sickle, FASTX-Toolkit, BBduk

RNAseq – Mapping Reads

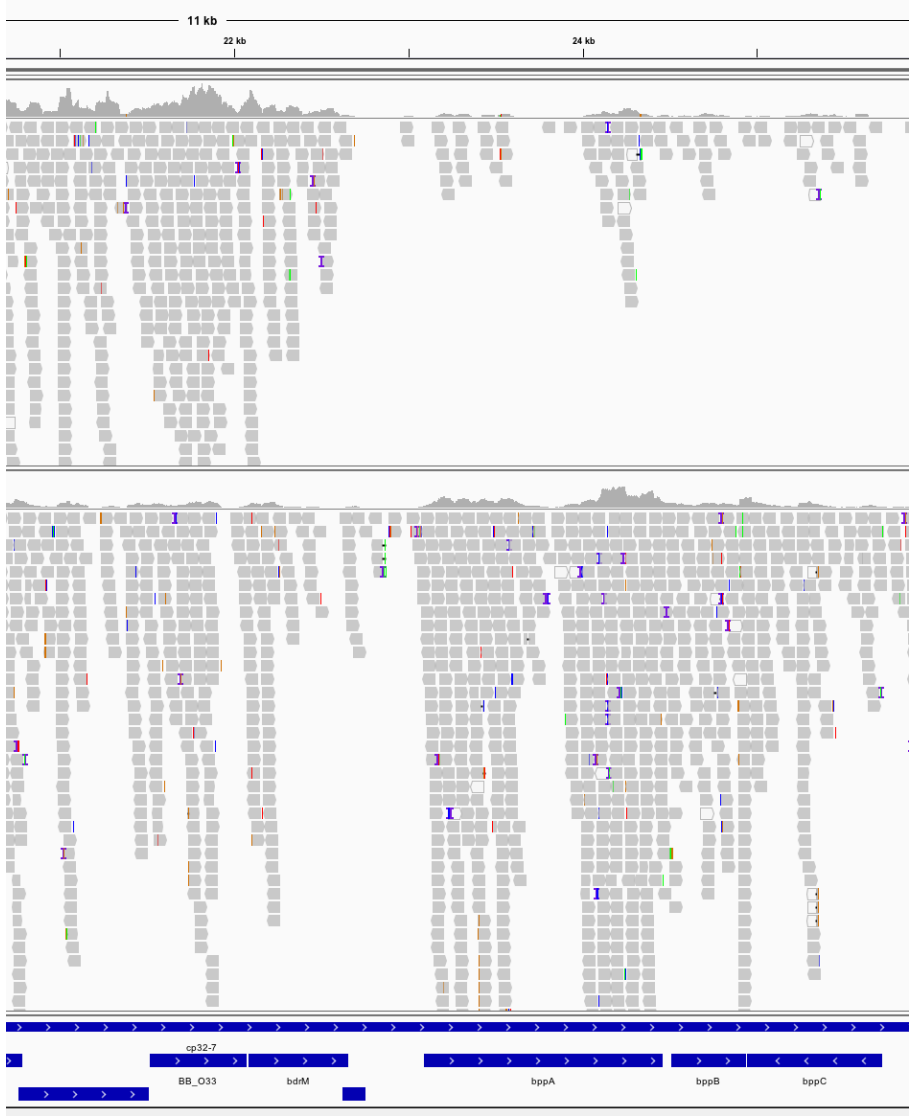
Design Experiment

RNA Preparation

Prepare Libraries

Sequence

Analysis



[Bowtie2](#) – great general use

Splice aware?

- [HISAT2](#)
- [BWA-MEM](#)
- [STAR](#) – gene counts as output

Pseudoalignment – very fast

- [Kallisto](#)
- [Salmon](#)

RNAseq – Alignment files – SAM/BAM

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Design Experiment

RNA Preparation

Prepare Libraries

Sequence

Analysis

SAM/BAM format

read_42 99 ref_genome 155 42 75M = 162 80 ATTTTGTTTT <CCCCGGGFF AS:i:0 XN:i:0

Col	Field	Type	Regexp/Range	Brief description
1	QNAME	String	`[!-?A-~]{1,254}`	Query template NAME
2	FLAG	Int	$[0,2^{16}-1]$	bitwise FLAG
3	RNAME	String	`\*|[!-()+-<>-~][!-~]*`	Reference sequence NAME
4	POS	Int	$[0,2^{31}-1]$	1-based leftmost mapping POSition
5	MAPQ	Int	$[0,2^{8}-1]$	MAPping Quality
6	CIGAR	String	`\*|([0-9]+[MIDNSHPX=])+`	CIGAR string
7	RNEXT	String	`\*|=|[!-()+-<>-~][!-~]*`	Ref. name of the mate/next read
8	PNEXT	Int	$[0,2^{31}-1]$	Position of the mate/next read
9	TLEN	Int	$[-2^{31}+1,2^{31}-1]$	observed Template LENgth
10	SEQ	String	`\*|[A-Za-z=.]+`	segment SEQuence
11	QUAL	String	`[!-~]+`	ASCII of Phred-scaled base QUALity+33

RNAseq – General Analysis Workflow

National Institute of Allergy and Infectious Diseases

Design Experiment

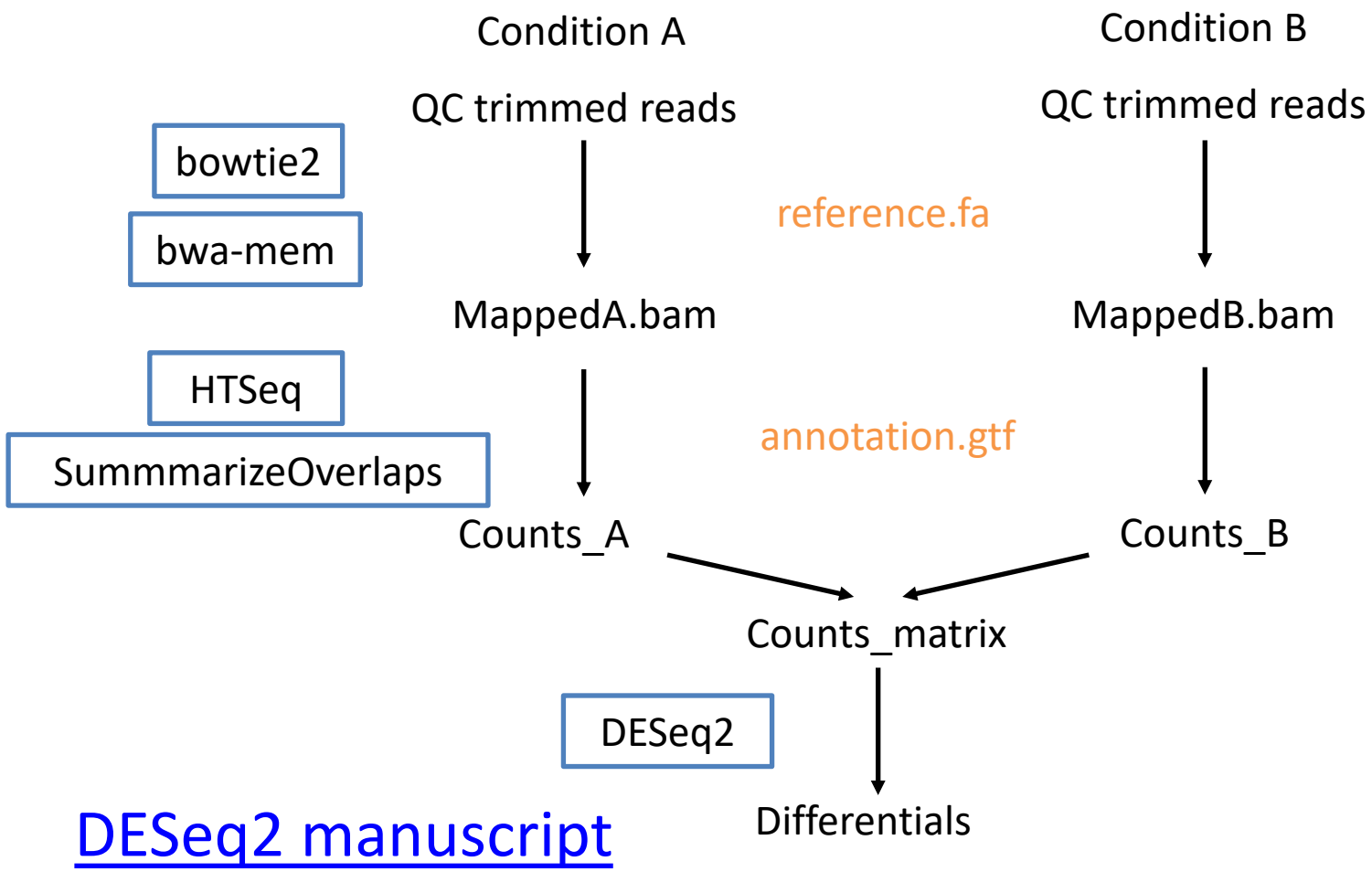
RNA Preparation

Prepare Libraries

Sequence

Analysis

Prokaryote/Eukaryote – gene level



DESeq2 manuscript

RNAseq – General Analysis Workflow

National Institute of Allergy and Infectious Diseases

Design Experiment

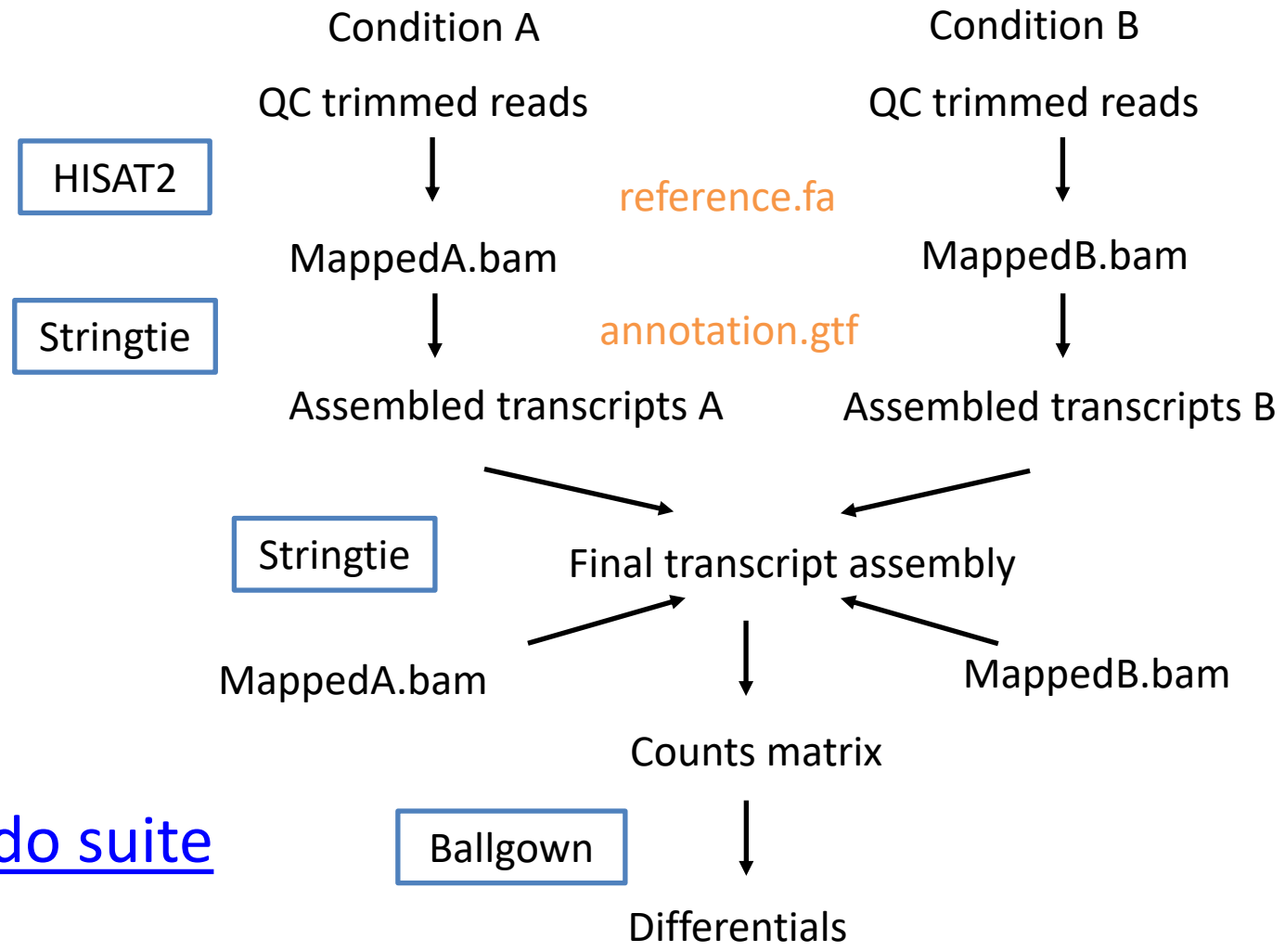
RNA Preparation

Prepare Libraries

Sequence

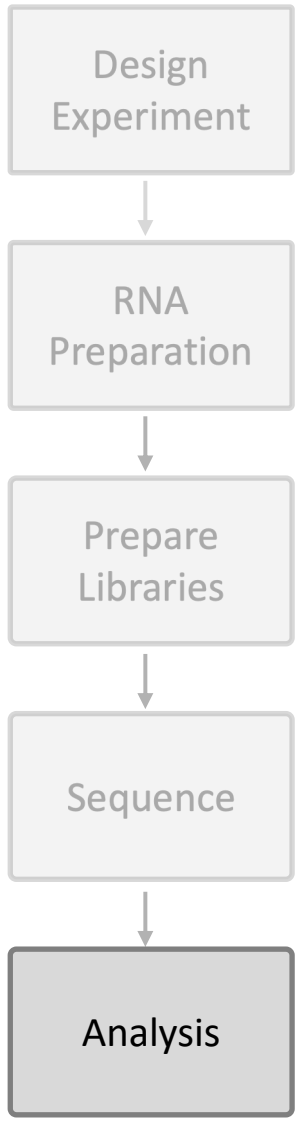
Analysis

Eukaryote – transcript level



[Tuxedo suite]

RNAseq – Differential Gene Expression



example RNAseq analysis pipeline with R

R is a free software environment for statistical computing and graphics

Rstudio is an integrated development environment (IDE) for R

Similar DESeq2 tutorial here

RNAseq – Differential Gene Expression w/ DESeq2

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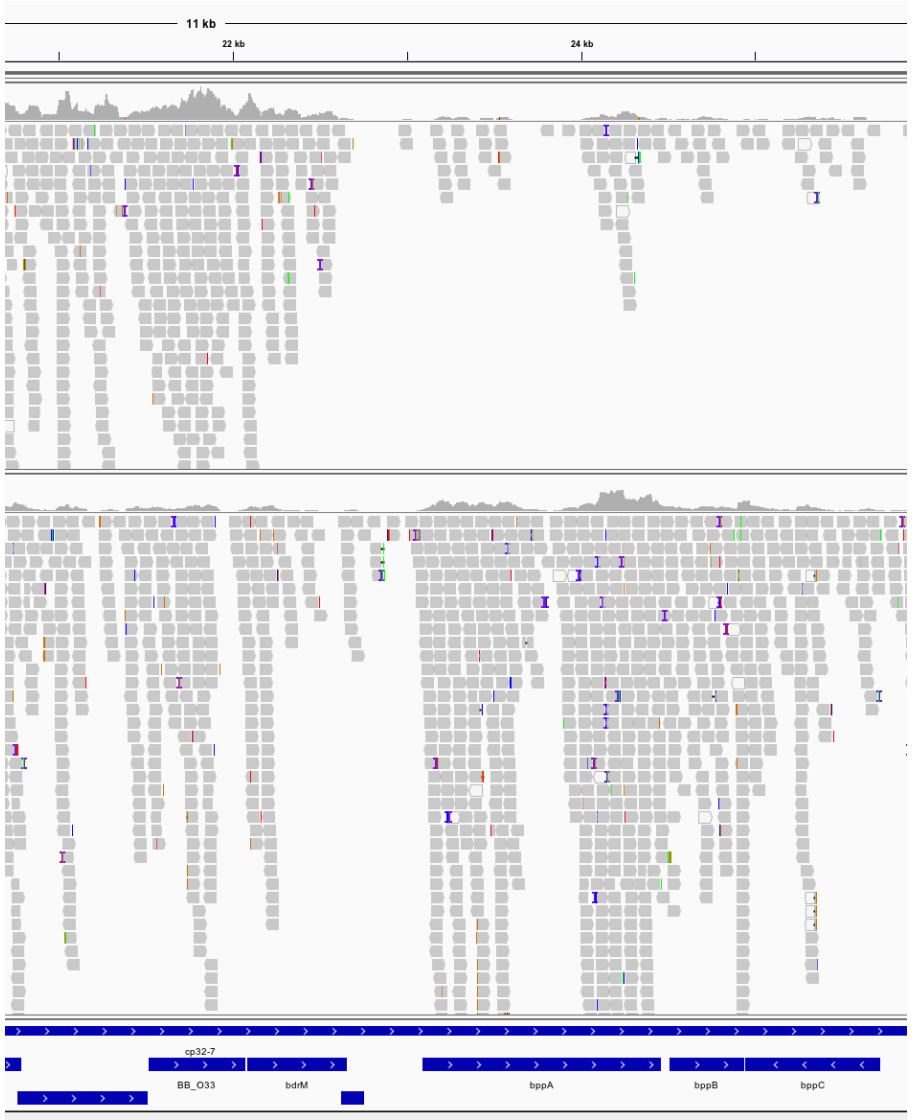
Design Experiment

RNA Preparation

Prepare Libraries

Sequence

Analysis



Simplest form, asking if 'count' between two treatments for a gene is not zero, reject null hypothesis

DESeq2 uses a generalized linear model where read counts are modeled using a Negative Binomial Distribution

- mean and variance are not equal (poisson)
- NB uses a gene-specific dispersion parameter

Wald test - estimated standard error of a log2 fold change to test if it is equal to zero

Likelihood Ratio Test (LRT) - useful for testing multiple terms at once. Conceptually similar to ANOVA

RNAseq – Differential Gene Expression Tool Choice

National Institute of Allergy and Infectious Diseases

Design Experiment

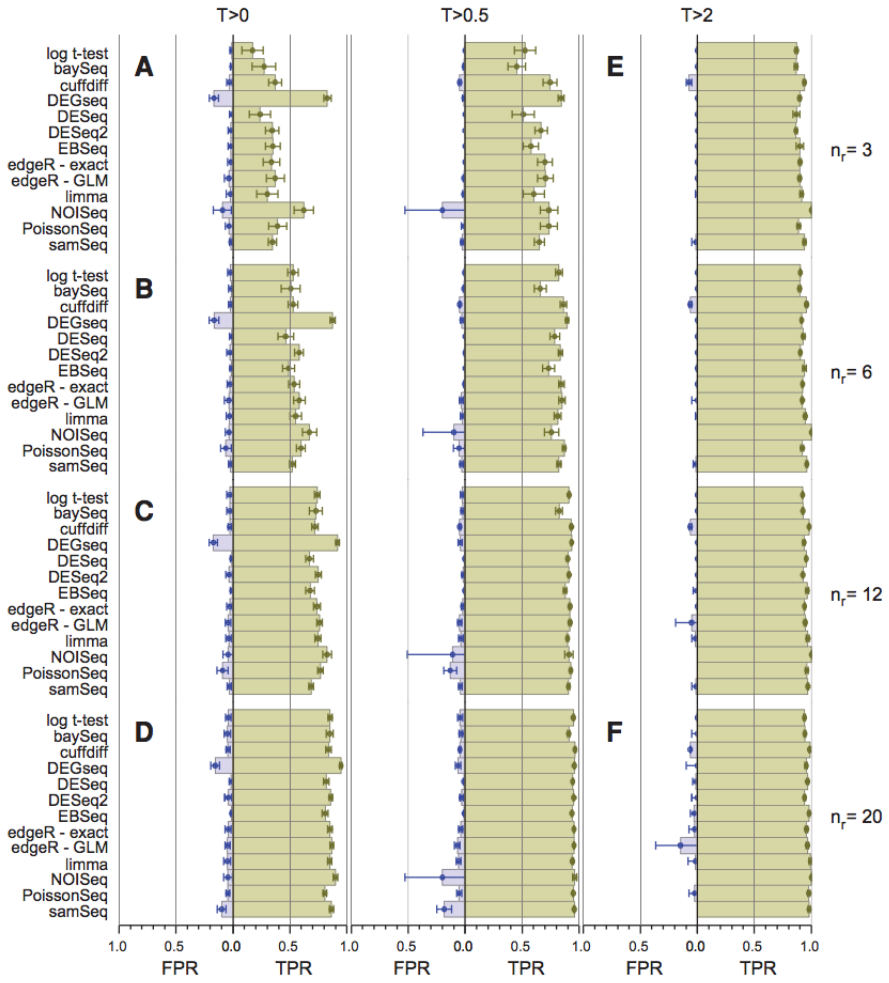
RNA Preparation

Prepare Libraries

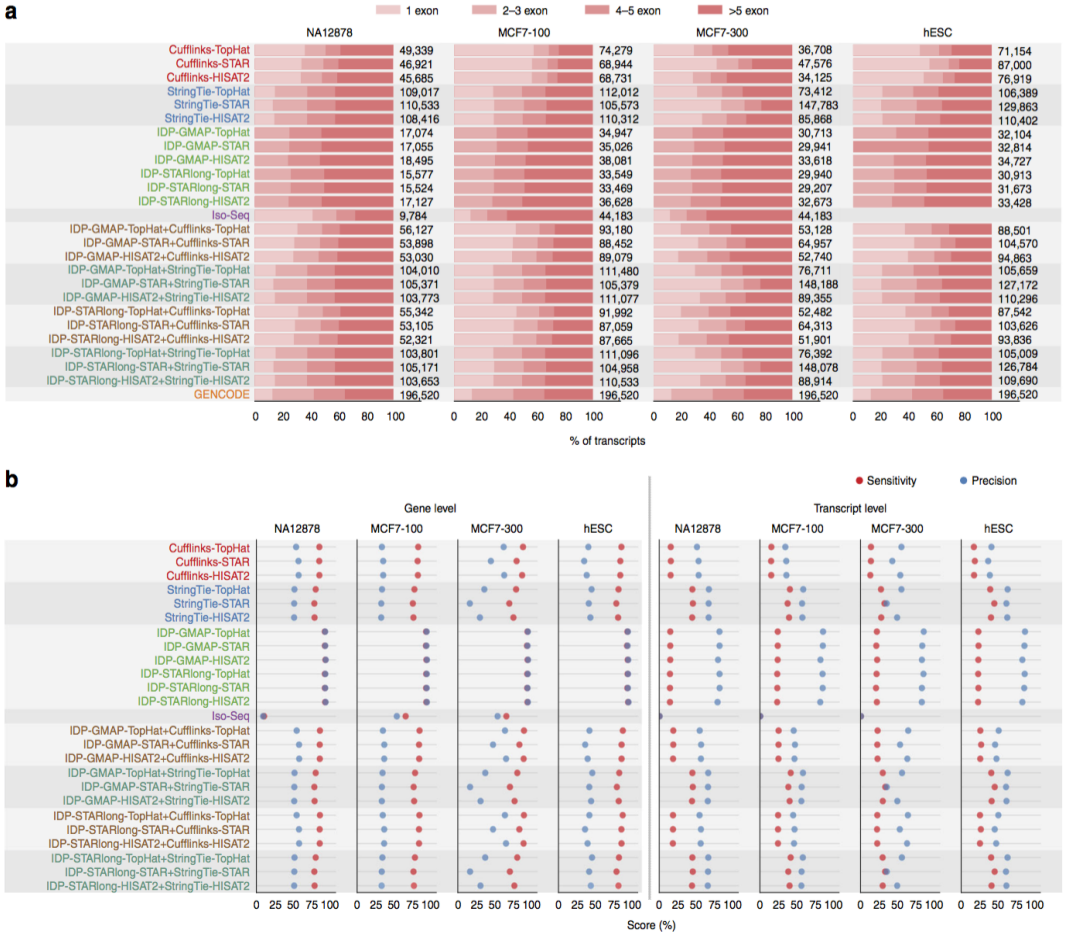
Sequence

Analysis

[Schurch et al.]



[Sahraeian et al.]



Pulling project code from GitHub

National Institute of Allergy and Infectious Diseases

Type the following in your favorite browser
github.com/niaid/ACE_Uganda_2021_RNAseq

Click on ‘Rstudio_server_instructions.txt’

RNAseq – R SummarizedExperiment

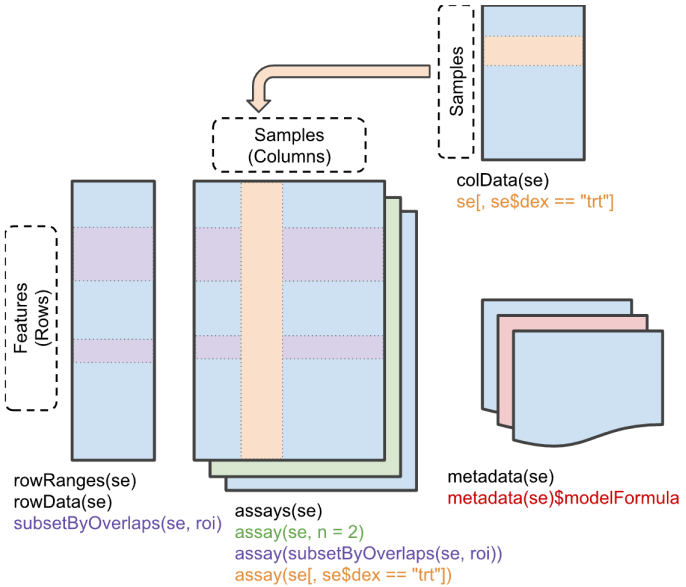
National Institute of Allergy and Infectious Diseases

Goals for example RNAseq analysis

1. Generate a matrix of counts of reads mapped to each gene for each sample
    1. SummarizedExperiment
    2. Coordination of metadata (experimental design) with the count matrix

2. Sample exploration and QC
    1. Look at potential batch effects

3. Differential Expression analysis



RNAseq – R SummarizedExperiment

National Institute of Allergy and Infectious Diseases

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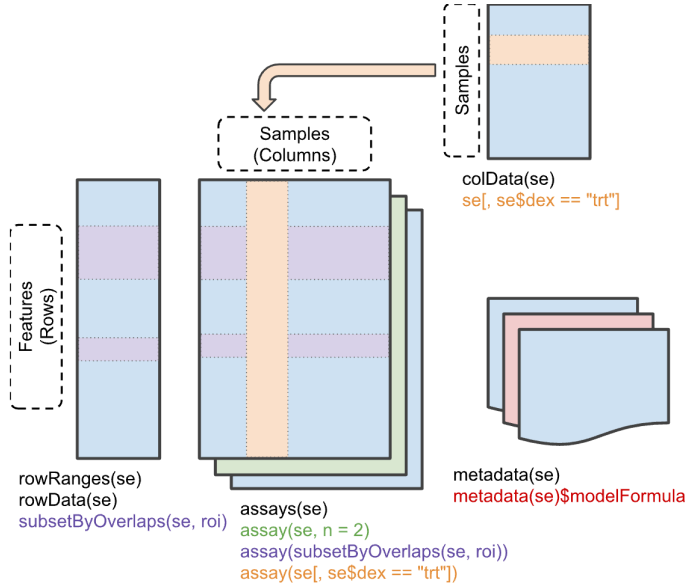

rowRanges(se)

gene	feature	start	stop
BB_002	exon1	2357	2987
BB_002	exon2	3045	4012
BB_002	exon3	4098	5765
BB_003	exon1	12987	13456
BB_003	exon2	14556	16777

Assay(se)

gene	con-1	con-2	con-3	con-4	trt-1	trt-2	trt-3	trt-4
BB_0002	136	145	131	101	260	169	217	322
BB_0004	459	287	391	118	295	289	206	331
BB_0005	780	591	724	237	471	450	338	470
BB_0006	1427	1393	1712	680	1330	1164	842	1280
BB_0007	959	603	775	420	708	777	402	483
BB_0008	113	84	85	27	88	100	75	82
BB_0009	92	64	56	14	49	53	35	52
BB_0010	244	210	271	214	437	367	283	308
BB_0011	543	372	515	170	423	544	264	265
BB_0012	187	112	125	64	106	142	90	114
BB_0013	81	30	39	15	46	35	31	54
BB_0014	330	243	247	78	154	164	117	209
BB_0015	1520	1239	1534	271	559	495	383	501
BB_0016	373	102	158	74	128	151	70	95
BB_0017	116	114	122	42	113	112	82	167

colData(se)

replicate	condition	time
con-1	ph76_C	2
con-2	ph76_C	2
con-3	ph76_C	4
con-4	ph76_C	4
trt-1	ph68_N	2
trt-2	ph68_N	2
trt-3	ph68_N	4
trt-4	ph68_N	4

RNAseq – R SummarizedExperiment

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Example GFF

```
##gff-version 3.2.1
LmjF.04	TriTrypDB	exon	1	1000	.	-	.	transcript_id "rna_LmjF.04.1"; gene_id "LmjF.04.1"; gene_name "LmjF.04.1";
LmjF.04	TriTrypDB	CDS	1	1000	.	-	0	transcript_id "rna_LmjF.04.1"; gene_id "LmjF.04.1"; gene_name "LmjF.04.1";
LmjF.04	TriTrypDB	exon	1001	2000	.	-	.	transcript_id "rna_LmjF.04.1001"; gene_id "LmjF.04.1001"; gene_name "LmjF.04.1001";
LmjF.04	TriTrypDB	CDS	1001	2000	.	-	0	transcript_id "rna_LmjF.04.1001"; gene_id "LmjF.04.1001"; gene_name "LmjF.04.1001";
LmjF.04	TriTrypDB	exon	2001	3000	.	-	.	transcript_id "rna_LmjF.04.2001"; gene_id "LmjF.04.2001"; gene_name "LmjF.04.2001";
LmjF.04	TriTrypDB	CDS	2001	3000	.	-	0	transcript_id "rna_LmjF.04.2001"; gene_id "LmjF.04.2001"; gene_name "LmjF.04.2001";
LmjF.04	TriTrypDB	exon	3001	4000	.	-	.	transcript_id "rna_LmjF.04.3001"; gene_id "LmjF.04.3001"; gene_name "LmjF.04.3001";
LmjF.04	TriTrypDB	CDS	3001	4000	.	-	0	transcript_id "rna_LmjF.04.3001"; gene_id "LmjF.04.3001"; gene_name "LmjF.04.3001";
LmjF.04	TriTrypDB	exon	4001	5000	.	-	.	transcript_id "rna_LmjF.04.4001"; gene_id "LmjF.04.4001"; gene_name "LmjF.04.4001";
LmjF.04	TriTrypDB	CDS	4001	5000	.	-	0	transcript_id "rna_LmjF.04.4001"; gene_id "LmjF.04.4001"; gene_name "LmjF.04.4001";
LmjF.04	TriTrypDB	exon	5001	6000	.	-	.	transcript_id "rna_LmjF.04.5001"; gene_id "LmjF.04.5001"; gene_name "LmjF.04.5001";
LmjF.04	TriTrypDB	CDS	5001	6000	.	-	0	transcript_id "rna_LmjF.04.5001"; gene_id "LmjF.04.5001"; gene_name "LmjF.04.5001";
LmjF.04	TriTrypDB	exon	6001	7000	.	-	.	transcript_id "rna_LmjF.04.6001"; gene_id "LmjF.04.6001"; gene_name "LmjF.04.6001";
LmjF.04	TriTrypDB	CDS	6001	7000	.	-	0	transcript_id "rna_LmjF.04.6001"; gene_id "LmjF.04.6001"; gene_name "LmjF.04.6001";
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LmjF.04	TriTrypDB	CDS	8001	9000	.	-	0	transcript_id "rna_LmjF.04.8001"; gene_id "LmjF.04.8001"; gene_name "LmjF.04.8001";
LmjF.04	TriTrypDB	exon	9001	10000	.	-	.	transcript_id "rna_LmjF.04.9001"; gene_id "LmjF.04.9001"; gene_name "LmjF.04.9001";
LmjF.04	TriTrypDB	CDS	9001	10000	.	-	0	transcript_id "rna_LmjF.04.9001"; gene_id "LmjF.04.9001"; gene_name "LmjF.04.9001";
```

Reads that map to a gene in GTF file are added to a tally of read counts for that gene

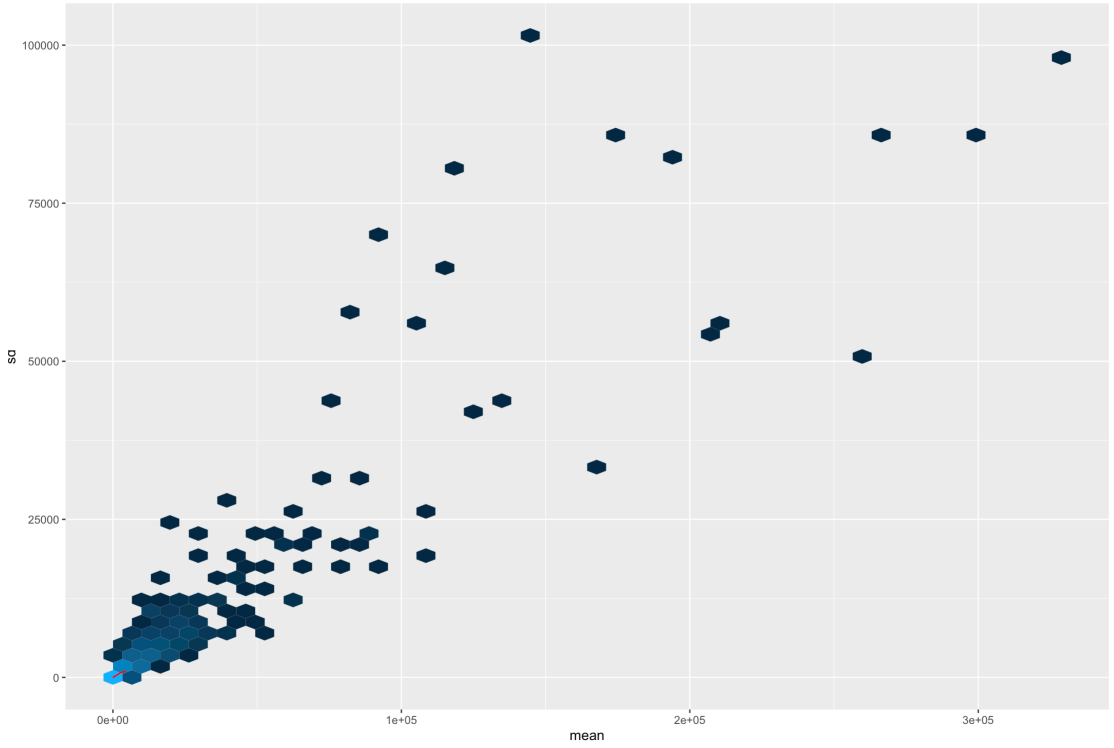
read_42 99 LmjF.04 4378 42 75M = 162 80 ATTTTGTTTT <CCCCGGGFF AS:i:0 XN:i:0

Coordinate read mapped to

RNAseq – R SummarizedExperiment

National Institute of Allergy and Infectious Diseases

Mean read counts vs SD - RAW



Mean read counts vs SD - VST