

# Hands-on: User-friendly Transcriptome Analysis Pipeline

- 1. From SandBox samples to sequenced reads
- 2. Setting up a new transcriptome analysis
- 3. How to read the MARS-seq report







# Bareket Dassa, June 2020



Before we start....

Ronald A. Fisher (1890-1962)

"To consult the statistician **after** an experiment is finished is often merely to ask him to conduct a post mortem examination. He can perhaps say what the experiment died of." (1938)

Think about your experimental design:

- 1. Biological question
- 2. Quality of the input material (method of RNA extraction)
- 3. Replicates
- 4. Batch effects
- 5. Coverage: how many reads?
- 6. Select library protocol, Paired- or single-end sequencing?



# Come and consult us **before** performing your experiment

# Bioinformatics unit NGS data analysis











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This training does not cover the bioinformatics theory behind RNA-seq analysis

Learn more with our:

Workshop: Introducing UTAP: User-friendly Transcriptome Analysis Pipeline (July 6th 2020) Course: An Introduction to deep-sequencing analysis for biologists 20203331 e-learning tool (to be released)



# Part 1:



# From SandBox samples to sequenced reads

where are my files?



# **Detailed guidelines:**

https://bbcunit.atlassian.net/wiki/spaces/BP/pages/104431617/From+samples+to+analyzed+NGS+data+UTAP



# Select your sequencing machine

	Home	Apps 👻	How To
The NGS workflow is describe	ed here.		
Sun Sep 8 09:19:13 2019			
Select your NGS machine:	•		
Questions And Support	NextSeq500 #1 NextSeq550 #2 NovaSeq6000-A INCPM		
<u>Library preparation:</u> Hadas Keren-Shaul (had	NovaSeq6000-B INCPM NextSeq500 INCPM das.keren-shaul@weizmann	i.ac.il)	
<u>SampleSheet:</u> Merav Kedmi ( <mark>merav.ke</mark> David Pilzer ( <mark>david.pilze</mark>	dmi@weizmann.ac.il) er@weizmann.ac.il)		
<u>NextSeq:</u> 脅 Levine ௴ Muriel Che 脅 INCPM ௴ Dana Rob	emla (muriel.chemla@weizn bins (dana.robbins@weizm	nann.ac.il) ann.ac.il)	)
Registration and access Irit Orr (irit.orr@weizma	to susanc: ann.ac.il)		
<u>Downloading data:</u> Vitaly Golodnitsky (vital	y.golodnitsky@weizmann.a	ic.il)	



**Warning:** Use NovaSeq only for unique **dual** indexing pooling combinations (unique i5 and i7 **indexes**). Index hopping or index switching is

a known phenomenon in NovaSeq. It causes incorrect assignment of libraries from the expected index to a different index (in the multiplexed pool).

# Select for demultiplexing

to get your reads separated into samples (according to sample barcodes provides in your SampleSheet)



NextSeq Run Additional Info	prmation
Run ID	<flow_cell_information></flow_cell_information>
User	<userid></userid>
PI	<pi></pi>
Want Bioinformatics Support	2 No If you want for the Bioinformatics Unit staff to process and analyze the data once the NextSeq run is done.
Want Demultiplexing or protocol 10X Genomics?	. In the second
Next step	



# **Example of MARS-seq Sample Sheet**

Α	В	С	D	E	F	G	Н	I. I.	J	K
#	Sample ID	rd1 barcode	rd1 barcode name	rd2 barcode name	rd2 barcode	i7 index barcode	i7 index name	i5 index barcode	i5 index name	userid
1	CTR_1			v3_gr1C2	GCAGTAG					sorek
2	CTR_2			v3_gr1B1	ACTCAGG					sorek
3	CTR_3			v3_gr1C1	ACACGTG					sorek
4	CTR_4			v3_gr1D1	TCTTCGA					sorek
5	CTR_5			v3_gr1E1	AGCACTG					sorek
6	KO_1			v3_gr1F1	AGTGCGT					sorek
7	KO_2			v3_gr1G1	AACGTTC					sorek
8	KO_3			v3_gr1H1	ATCTGTC					sorek
9	KO_4			v3_gr1A2	GTATGCG					sorek
10	KO_5			v3_gr1B2	ATCTGCA					sorek
11	WT_1			v3_gr1C2	GCAGTAG					hadask
12	WT_2			v3_gr1D2	CAGTACG					hadask
13	WT_3			v3_gr1E2	TGCACAA					hadask

#### Give meaningful names for your samples!

Note: Valid characters for sample names are A-Z a-z 0-9 . \_ -

Don't use special characters such as " ' `?, ; + = @ # \$ % ^ & () [] {} <> / \ in sample names.

Don't use Hebrew, Arabic, Chinese or any character-set other than English (Roman alphabet)

Test your SampleSheet!

# **Example of MARS-seq Sample Sheet**

#### It's recommended to test in advance your SampleSheet or MARSseq file CLICK HERE)

Α	В	С	D	E	F	G	Н	I	J	K
#	Sample ID	rd1 barcode	rd1 barcode name	rd2 barcode name	rd2 barcode	i7 index barcode	i7 index name	i5 index barcode	i5 index name	userid
1	CTR_1			v3_gr1C2	GCAGTAG					sorek
2	CTR_2			v3_gr1B1	ACTCAGG					sorek
3	CTR_3			v3_gr1C1	ACACGTG					sorek
4	CTR_4			v3_gr1D1	TCTTCGA					sorek
5	CTR_5			v3_gr1E1	AGCACTG					sorek
6	KO_1			v3_gr1F1	AGTGCGT					sorek
7	КО_2			v3_gr1G1	AACGTTC					sorek
8	КО_3			v3_gr1H1	ATCTGTC					sorek
9	КО_4			v3_gr1A2	GTATGCG					sorek
10	KO_5			v3_gr1B2	ATCTGCA					sorek
11	WT_1			v3_gr1C2	GCAGTAG					hadask
12	WT_2			v3_gr1D2	CAGTACG					hadask
13	WT_3			v3_gr1E2	TGCACAA	Test here		a SampleSh	eet	
14	WT+treatme	ent_1		v3_gr1F2	CGTAACT	lest liere	your Nextoe	q bampicon		I
15	WT+treatine	ent_2		v3_gr1G2	AGCTCAA	Please fix	and resubmi	t		
16	WT+treatme	ent_3		v3_gr1H2	ATAACCG	Illegal cha	racters Sam	ole 'WT+treati	ment 1'	
17	mutant_1			v3_gr1A3	TGTCACG	Illegal cha	racters Sam	ole 'WT+treat	ment_2'	
18	mutant_2			v3_gr1B3	TTCCTGA	- Illegal cha	ractors Sam	olo WT+troat	mont_2	
19	mutant_3			v3_gr1C3	GGATCTA	Duplicator	d barcada C(			
20	Mutant-trat	ment_1		v3_gr1D3	TACCAGT	Duplicated	a barcoue Go	AGTAG OF	/IK_1, WI_	
21	Mutant-trat	ment_1		v3_gr1E3	GGAGACT	File to test	Choose Fil	e Mars-seg us	are viev	
22	Mutant-trat	ment_1		v3_gr1F3	AGCTAGT	1 110 10 1051	Choose I II	e Mais-seq_us	613.4134	
							Submit			

When sharing a flow cell with other users:

- Use unique sample barcodes
- Do not mix sequencing protocols in one run
- Do not overload

# **Retrieving your NGS data:**

You will receive an email from the Bioinformatic-Unit with:

1. A link to your Raw (Bcl and Fastq) output files http://stefan.weizmann.ac.il/fqc/RUN ID

# Data is temporarily stored only for 3 months

Download your data via WGET option on UNIX, or from your web browser.

# 2. A link to the first QC report



A sequencing run typically takes a few hours or more to complete, depending on the number of samples

# Explore the first QC report



#### **Flowcell Summary**

#### Clusters (Raw) Clusters (PF) Yield (MBases)

269,921,898 262,607,862 19,696

#### Basic parameters per sample

Sample	Index	# PF Clusters 🕄	% Clusters 🛈 per sample	Yield 🛈 (MBases)	%≥Q30 <mark>1)</mark> FastQC Analysis
1	CAAGGCGA	14,844,567	5.65	1,113	99.07 R1 😫 🔎
2	GACGCTAT	15,428,025	5.88	1,157	99.08 R1 😫 🔎
3	ACTTCTTC	46,535,122	17.72	3,490	99.08 R1 😫 🔎
4	CCTAGAAT	50,891,981	19.38	3,816	99.03 R1 😫 🔎
5	TGGTAACG	25,363,955	9.66	1,902	99.13 R1 😫 🔎
6	CATCAGAC	33,953,457	12.93	2,547	99.02 R1 😫 🔎
7	GTGCGTAA	20,900,400	7.96	1,569	99.06 R1 😫 🔎
8	CTATTCAA	117	0.00	0	97.44 R1 😫 🔎
Undetermined Indices	Indetermined	54,690,238	20.82	4,102	

# Explore the first QC report

#### General QC for run

#### Sequence protocol: Single-read

#### Quick Navigation

Sequence quality #PF reads Flowcell Summary Basic parameters per sample

# See here a more comprehensive report of MultiQC software

A modular tool to aggregate results from bioinformatics analyses across many samples into a single report.

Report generated on 2019-02-28, 16:17 based on data in: //data/fastq/190219\_NB501465\_0472\_AHT7WwBGX9/FastQC

Welcome! Not sure where to start? Watch a tutorial video (6:06)

#### **General Statistics**

Copy table Configure Columns III Plot Showing 84/84 rows and 4/5 columns.				
Sample Name	% Dups	% GC	Length	M Seqs
MB38-005_AllPrep_R1	9.0%	49%	75 bp	3.0
MB38-005_AllPrep_R2	97.8%	47%	15 bp	3.0
MB38-005_Rneasy_R1	5.4%	53%	75 bp	0.7
MB38-005_Rneasy_R2	91.7%	51%	15 bp	0.7

# Per base seauence quality

#### First QC report

Demo:

http://stefan.weizmann.ac.il/fqc/180708\_NB551168\_0156\_A H2F77BGX7/

# From sequencing to analyzed data



# **Detailed guidelines:**

https://bbcunit.atlassian.net/wiki/spaces/BP/pages/104431617/From+samples+to+analyzed+NGS+data+UTAP



# Part 2: Setting up a transcriptome analysis with UTAP



# **UTAP: User-friendly Transcriptome Analysis Pipeline**



# **1. Setting up a new transcriptome analysis**

Before you start- please prepare in advance:

- 1. An **account** (userID) on Wexac computer cluster
- 2. A "**Collaboration**" folder with read and write permissions for the Bioinformatics unit
- 3. Sufficient free **storage** space on Wexac (> 400Gb).

Transfer demultiplexed sequencing data (fastq files) to your Wexac Collaboration folder

• UTAP Pipeline website: <u>http://ngsbio.wexac.weizmann.ac.il</u> OPEN WITH CHROME BROWSER

# UTAP Manual

https://bbcunit.atlassian.net/wiki/spaces/BP/pages/509214758/UTAP+guidelines-+User-friendly+Transcriptome+Analysis+Pipeline

# Setting up a new transcriptome analysis

# Select the type of analysis

BBCU - NGS PIPELINES	User Datasets	Run pipeline	Help	userID Logout
	Run a	analysis peline from the lis	t.	
	Choose pipeline: Transcrit Demultij Demultij Demultij	ptome RNA-seq ptome Mars-seq plexing_from_RU plexing_from_FA plexing_from_BC	VID STQ L	

Fill in a project name, select the reference genome and annotation for which the reads will be aligned to

Run analysis The input files need to be under Collaboration folder in Wes	ac server. See help for more details.		
Choose pipeline:	Chosen pipeline: Project name: date(D/M/Y)_time(H Input folder: Genome: Annotation: Output folder: User email: Deseq run: Advanced parameters Run analysis	Transcriptome Mars-seq	Transcriptome Mars-seq

# Select the input folder

Pipeline:	Transcriptome Mars-seq				
Project name:					
Input folder:					
Genome:		•			
Annotation:	▼				
Output folder:					
Deseq run:	No Deseq 🔹	_			
Run analysis	FileBrowser > NGS_rt	un_ID_1			NEW FOLDER UPLOAD
		Search			By Date
	Select Folder	folder1	SIZE —	DATE Nov. Ct 7, 2017	Any Date Today Past 7 days
	Select -	a.fa	340 bytes	Nov. 🖋 Cł 7, 2017	This year
	Select	NGS_run_ID_2	915 bytes	Nov. Ct 7, 2017	All Folder
	Select Folder	NGS_run_ID_3	240 bytes	Nov. 🖋 Cł 7, 2017	Image Document Video
	Select Folder	NGS_run_ID_4	84 bytes	Nov. 🖋 Cł 6, 2017	Audio

# Differential gene expression analysis with DESeq2 package

Deseq run:	Run Deseq	T			
				Add Category	Remove Category
Filter samples (type part o	f the name)		*	1w	
24h_DMSO 24h_etop		<b>^</b>	>	1w_DMSO 1w_etop	*
			<		
			•		
		U			-
		ſ	•	Level 2 name	
			<u> </u>		*
		l	"		-
		Ŧ			
Submit for runing in end of	f the sequencing				

Create categories for the treatments that you would like to compare

# **Relate to batch effects**



# **Run the pipeline**



# UTAP outputs are available at:

### 1. A link to the report sent by mail

2. UTAP pipeline website

	AP - NGS PIPELINES	User Datasets	Upload data	Run pipeline	Help		
--	--------------------	---------------	-------------	--------------	------	--	--

#### Analyses List:

	Name	Run status	Pipeline	Created	
Delete	20180814_114552_RNA-seq-example	SUCCESSFUL	Transcriptome RNA-seq	Oct. 29, 2018, 4:08 p.m.	Run Deseq again with other parameters
Delete	⇒ 20190515_133007_RNA-seq-example_fcfc	SUCCESSFUL	Transcriptome RNA-seq Deseq	May 15, 2019, 1:30 p.m.	
Delete	⇒ 20190509_172904_RNA-seq-example_t	SUCCESSFUL	Transcriptome RNA-seq Deseq	May 9, 2019, 5:29 p.m.	
Delete	⇒ 20190408_164100_RNA-seq-example_pippo	SUCCESSFUL	Transcriptome RNA-seq Deseq	April 8, 2019, 4:41 p.m.	

# 3. Collaboration folder on WEXAC:



# 20180814\_114552\_RNA-seq-example

Name:	20180814_114552_RNA-seq-example
Job-id:	2
Status:	SUCCESSFUL
Pipeline:	Transcriptome RNA-seq
Created:	Oct. 29, 2018, 4:08 p.m.
Run by:	testuser
Results:	Results
Parameters:	Parameters file
Run Deseq again with other	parameters
Delete	



# Part 3: How to read the MARS-seq report

Please regard this analysis as a good starting point and not an end result!







MARS-seq example report: https://bip.weizmann.ac.il/mars-seq

# Total number of reads for each sample in raw data

There is **always** an unequal distribution of reads per samples on a flowcell



# Summary of the number of reads for each sample in each step of the pipeline



Genomics regions to which the reads (raw data) are mapped



# **Top highly-expressed genes**

(above 5% of total expression)

#### The fraction of reads from the genes with the most counts



very highly expressed genes can dominate the total lane count and skew the expression analysis

# **Explore samples Correlation**

Heatmap of Pearson correlation coefficients between gene expression values of each sample



# **Explore samples Correlation in a dendrogram**



# **PCA-** Principal component analysis

Dimensionality reduction to assess overall similarity between samples



# **Differential Expression Analysis**

Differential expression analysis is performed using DESeq2. Thresholds for **significant differential expressed genes** for each comparison:

|log<sub>2</sub>FoldChange| >= 1

rest

• padj <= 0.05

\_\_\_\_\_

baseMean >= 5

									_			
Comparison	Factor		Α	в	Formul	a	Padj correcte	d by fdrtoo	ы	Plots	DE Ge	nes
control_vs_LPS	control_or_l	PS	contro	I LPS	control_	_or_LPS	FALSE			link	link	
		Со	mparis	son wt	-rest_v	vs_dko-r	est					
		Show	20 v entries					Search:				
			Comparison	Gene 🍦	baseMean	log2FoldChange 🔷	linearFoldChange 🔷	pvalue 🔷	padj 🔶	pass 🝦	Direction 🔷	Plot 🔶
			All	4	AI	All	All				4	
		1	wt- rest_vs_dko- rest	BC018473	77.8	-8.042	-263.562261359103	2.1e-13 9	.6e-13	yes	down	••
		2	wt- rest_vs_dko- rest	Icam1	200	4.165	17.9386572484561	4e-23 9	.6e-13	yes	up	••
		3	wt- rest_vs_dko- rest	St6galnac2	64.2	-5.115	-34.6552014568296	3.5e-17 9	.6e-13	yes	down	••
		4	wt- rest_vs_dko- rest	Ddx3y	61.2	-3.699	-12.9870332956365	1e-11 5	.4e-11	yes	down	••
		5	wt- rest vs dko-	Xist	49	4.404	21,1707430385299	4.2e-9	8.7e-8	Ves	up	8

# Volcano plots An interactive scatter plot of significance versus fold-change



# Hierarchical clustering heat map of differentially expressed genes

using the genes expression values rld (log2 normalized)



# Links to functional enrichments analysis



To perform functional enrichments, you can try one or more of the following websites: Intermine, Reactome, GeneAnalytics from GeneCards<sup>(R)</sup> or STRING. You can also use the links below to send the differentially expressed genes directly to Intermine (In the first time click on the button twice to get the correct page.:

Show	10 · entries	Search:			
	Comparison	🔶 pass 🍦	Direction 🔶	Number of genes 🍦	InterMine 🔶
1	control_vs_LPS	yes	ир	449	Send
2	control_vs_LPS	yes	down	596	Send

# A few words on interpretation of p Value histograms



0.00

0.25

0.50

pvalue

0.75

1.00

# Additional documentations in the report

- Bioinformatics pipeline methods
- Links to results
- Quantification of data

### See excel

#### Quantification data

Quantification data including: raw counts, normalized counts and rld (log normalized counts) and pairwise deseq2 statistics can be downloaded here.

# Links to results

Sequences from folder: /home/labs/mosheoren/Collaboration/anat\_rna\_seq/180320\_D00257\_0307\_BCBYFNANXX/fastq

Output

folder: /home/labs/mosheoren/Collaboration/anat\_rna\_seq/180320\_D00257\_0307\_BCBYFNANXX/20.8.18\_anatger1/20180820\_140229\_20\_8\_18\_anatger1\_transcriptome\_RNA-seq/

Statistics regarding the number of reads for each sample for various steps of the pipeline can be downloaded from here.

Raw counts can be downloaded from here.

Normalized counts can be downloaded from here. Commands log can be downloaded from here.

# **Results file content**

- For each sample, 3 columns:
  - Raw read count
  - Normalized normalized read counts to the sample library size
  - Rld transformed normalized read counts

Count values for a gene can be zero in some conditions (and non-zero in others). For other downstream analyses – e.g. for visualization or clustering – it is useful to work with transformed versions of the count data:

y=log2(N+C)

Where:

N represents the count values C is a positive constant.

											Deseq_all_	results_add.	dsx - Excel				111			
	File Ho	ome Inse	rt Page La	yout For	mulas	Data Rev	iew Vie			what you wa	ent to do									
Pa	Cut	ry + mat Painter	Calibri B I <u>U</u> -	* 11 	• A* A*		8⁄- н	• 🔐 v	Vrap Text Aerge & (	Center ~	General \$ - %	, 3.3	Conditio Formattin	nal Format g • Table	As Good	1	Bad Neutral	4 4 1	Insert Dela	ete Format
	Clipboar	d ra		Font	5		Aligr	ment		5	Numb	ber r			Stj	les			Cel	(Is
	5.0	÷																		
A:	1	• I L ×	√ fx																	
l,			6	0	c		0				×		м	N	0	0	0	0		T
1	A	D		0	c	· ·	0			,	N		m	IN .		,	ų	n	3	
1		Sara_1.ra S w v	iara_2.ra Sar v vw	a_3.ra Sara	a_4.ra Sa	ra_5.ra Sara	_6.ra Sara	_7.ra Sar	a_8.ra	Sara_1.n ormalize d	Sara_2.n ormalize d	Sara_3.n ormalize d	Sara_4.n ormalize d	Sara_5.n ormalize d	Sara_6.n ormalize d	Sara_7.n ormalize d 💌	Sara_8.n ormalize d	Sara_1.rl	Sara_2.rl	Sara_3.rl
2	061000982	49	17	10	10	18	16	17	23	29.35254	13.76905	14.85645	7.576165	19.52178	13.02356	18.27305	37.01498	4.464856	4.039799	4.082922
3	0610009L1	13	42	6	6	3	6	16	1	7.787408	34.01765	8.913868	4.545699	3.253631	4.883834	17.19817	1.609347	3.106418	3.852157	3.154843
1	061000903	31	17	16	12	22	22	20	8	18.56997	13.76905	23.77031	9.091398	23.85996	17.90739	21.49771	12.87478	4.147422	3.99994	4.273903
5	0610010FC	18	6	1	14	14	6	11	1	10.78256	4.859665	1.485645	10.60663	15.18361	4.883834	11.82374	1.609347	3.008888	2.768754	2.632879
5	0610012G	221	164	82	224	217	243	209	134	132.3859	132.8308	121.8229	169.7061	235.346	197.7953	224.6511	215.6525	7.176411	7.181088	7.105588
7	0610030E2	133	100	77	144	56	65	63	28	79.67117	80.99442	114.3946	109.0968	60.73444	52.90821	67.71779	45.06172	6.27207	6.286016	6.592576
8	0610037L1	141	93	96	123	136	176	120	85	84.46342	75.32481	142.6219	93.18683	147.4979	143.2591	128.9863	136.7945	6.564466	6.466053	7.051163
9	061004081	9	3	0	7	2	5	3	0	5.391282	2.429832	0	5.303315	2.169087	4.069862	3.224657	0	1.614014	1.481825	1.376172
0	1110002J0	3	0	0	0	2	7	2	2	1.797094	0	0	0	2.169087	5.697807	2.149771	3.218694	0.837445	0.740162	0.752035
1	1110002L0	28	20	3	15	12	16	3	10	16.77288	16.19888	4.456934	11.36425	13.01452	13.02356	3.224657	16.09347	3.686895	3.665953	3.258091
2	111000200	0	0	13	0	0	0	0	0	0	0	19.31338	0	0	0	0	0	0.437564	0.445093	1.317435
100	111000251	i 9																		
3	1110003F1		/	6	6	5	5	0	3	1.797094	5.669609	8.913868	4.545699	5.422718	4.069862	0	4.828041	1.949057	2.137164	2.250035

# Results file: statistics for each comparison

Control_Treatment.	Control_Treatment	Control_Treatment	Control_Treatment.	Control_Treatment	Control_Treatment.
baseMean	.log2FoldChange	.pvalue	padj	.pass	Direction

- Control\_Treatment.baseMean mean (average) normalized read counts of all samples
- Control\_Treatment.log2FoldChange Fold change is a measure of the ratio of means of two populations (i.e control and treatment). Log2(2)=1
- Control\_Treatment.pvalue measures statistical significance of the difference of the two populations.

If you have, 10,000 genes, then

you expect 500 of them to have p<0.05 just by chance.

- Control\_Treatment.padj statistically significant as part of the multiple comparison testing (many genes).
- Control\_Treatment.pass Yes or No –

passing threshold: baseMean >= 5,  $|\log 2FoldChange| \ge 1$  and  $padj \le 0.05$ 

• Control\_Treatment.Direction – Up or Down



# LIFE SCIENCE CORE FACILITIES

# Support for other issues:

<u>Library preparation:</u> Hadas Keren-Shaul (hadas.keren-shaul@weizmann.ac.il)

<u>SampleSheet:</u> Merav Kedmi (merav.kedmi@weizmann.ac.il) David Pilzer (david.pilzer@weizmann.ac.il)

#### NextSeq:

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Registration and access to susanc: Irit Orr (irit.orr@weizmann.ac.il)

<u>Downloading data:</u> Vitaly Golodnitsky (vitaly.golodnitsky@weizmann.ac.il)

Learn more with our:

Workshop: Introducing UTAP: User-friendly Transcriptome Analysis Pipeline (July 6th 2020) Course: An Introduction to deep-sequencing analysis for biologists 20203331 e-learning tool (to be released)

Bioinformatics unit NGS data analysis



Noa

Wigoda



Ester

Feldmesser



Dena

Leshkowitz

Unit head





Bareket

Dassa



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