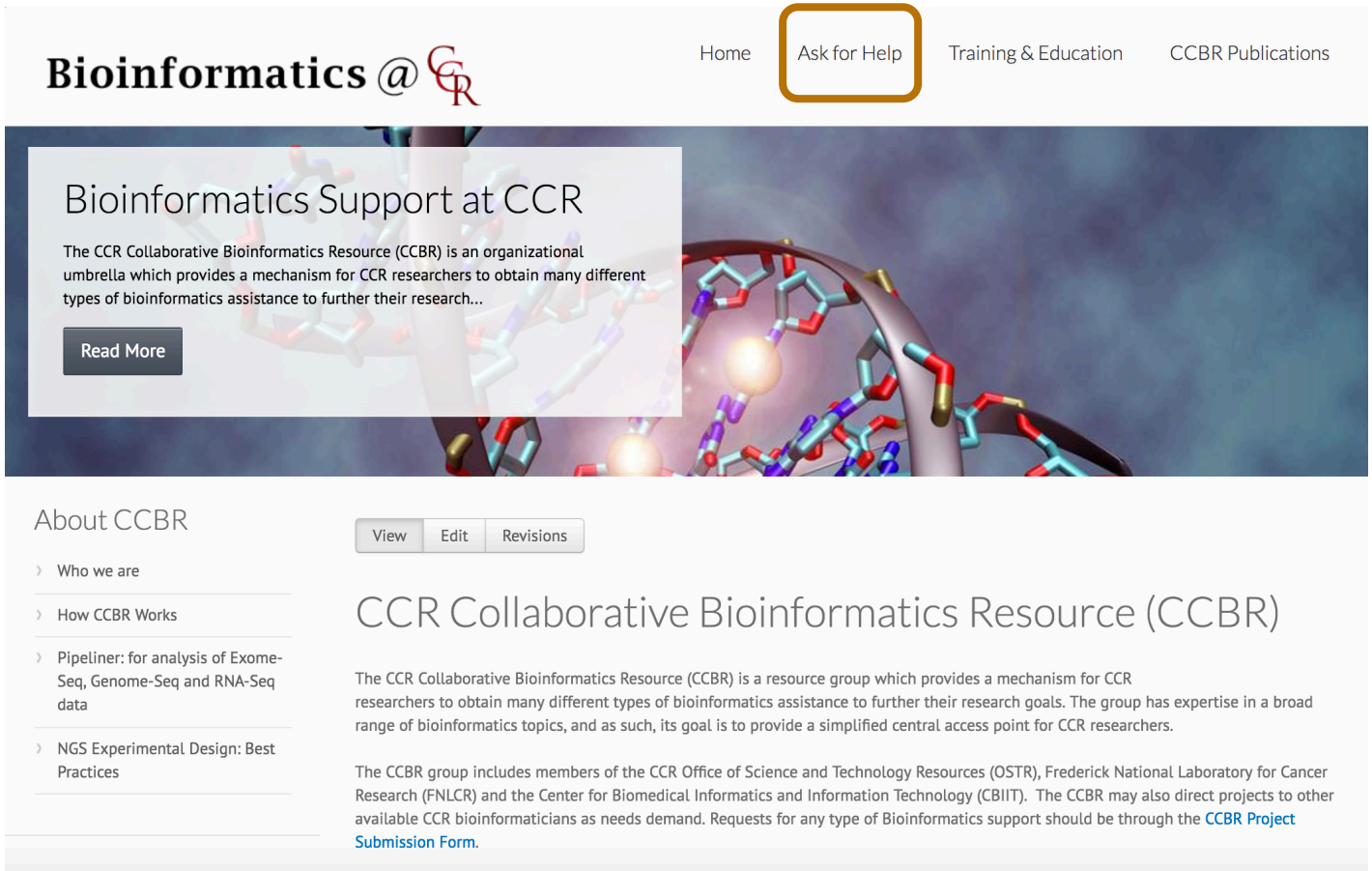
CCR Collaborative Bioinformatics Resource (CCBR)

INTRODUCTION TO NGS DATA ANALYSIS

BTEP SERIES 2017

bioinformatics.cancer.gov



CCBR support includes:

Consulting on experimental design, help with analysis and interpretation of biological data produced by large-scale genomics technologies including Next-generation sequencing (RNA-Seq, Exome-Seq, ChIP-Seq, Whole genome Sequencing), and microarrays

Support for the development of methods for new technologies provided by the Office of Science and Technology Resources (OSTR)

Provide training classes to CCR scientists focusing on software used in the analysis of their own data

CCBR Members

Office of Science and Technology Resources (OSTR)

Maggie Cam (Head)

Center for Biomedical Informatics and Information Technology (CBIIT)

Chunhua Yan

Ying Hu

Richard Finney

Frederick National Laboratory of Cancer Research (Leidos)

Parthav Jailwala (Manager)

Fathi Elloumi

Justin Lack

Bong-Hyun Kim

George Nelson

Alexei Lobanov

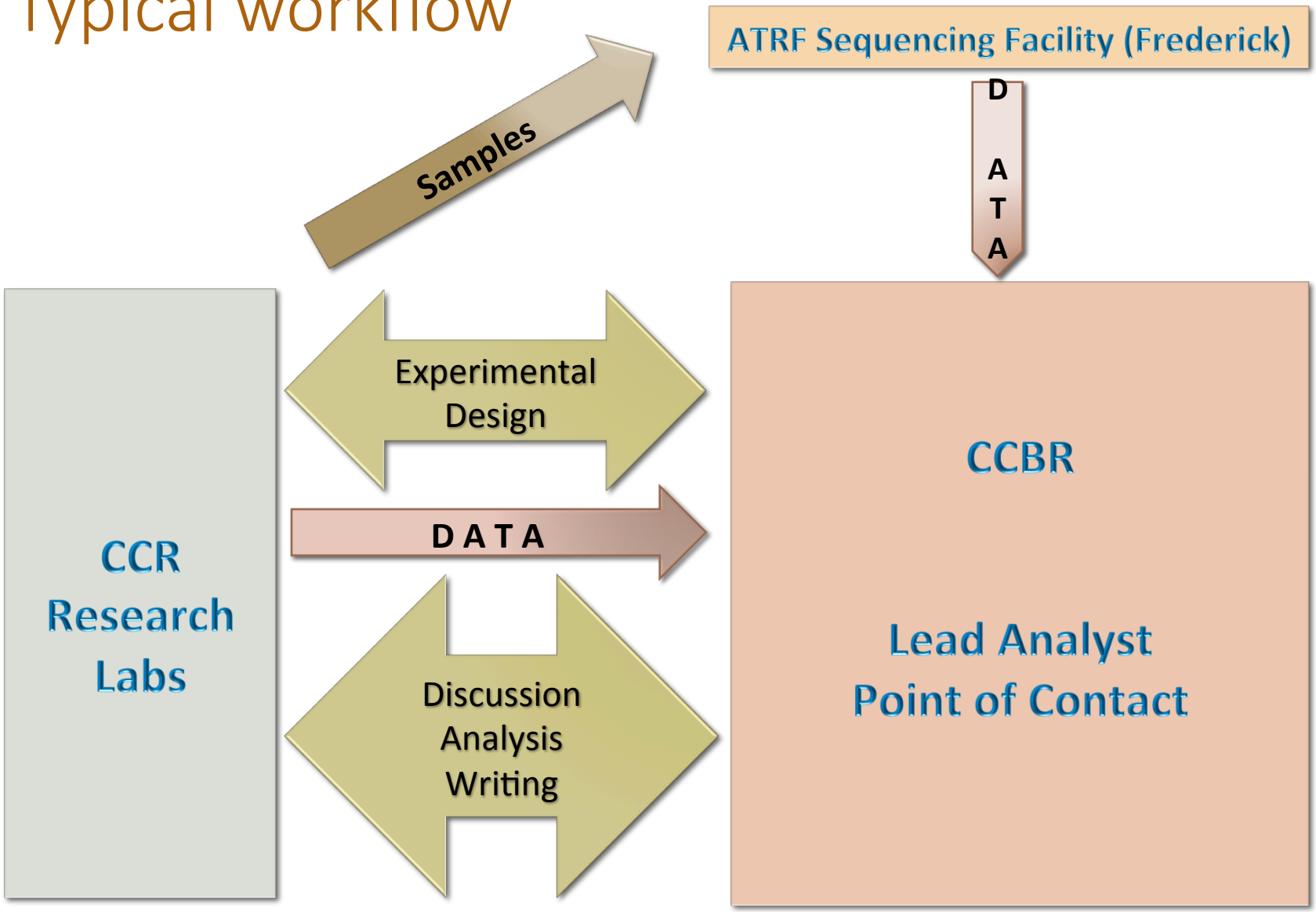
Jack Chen

Ashley Walton

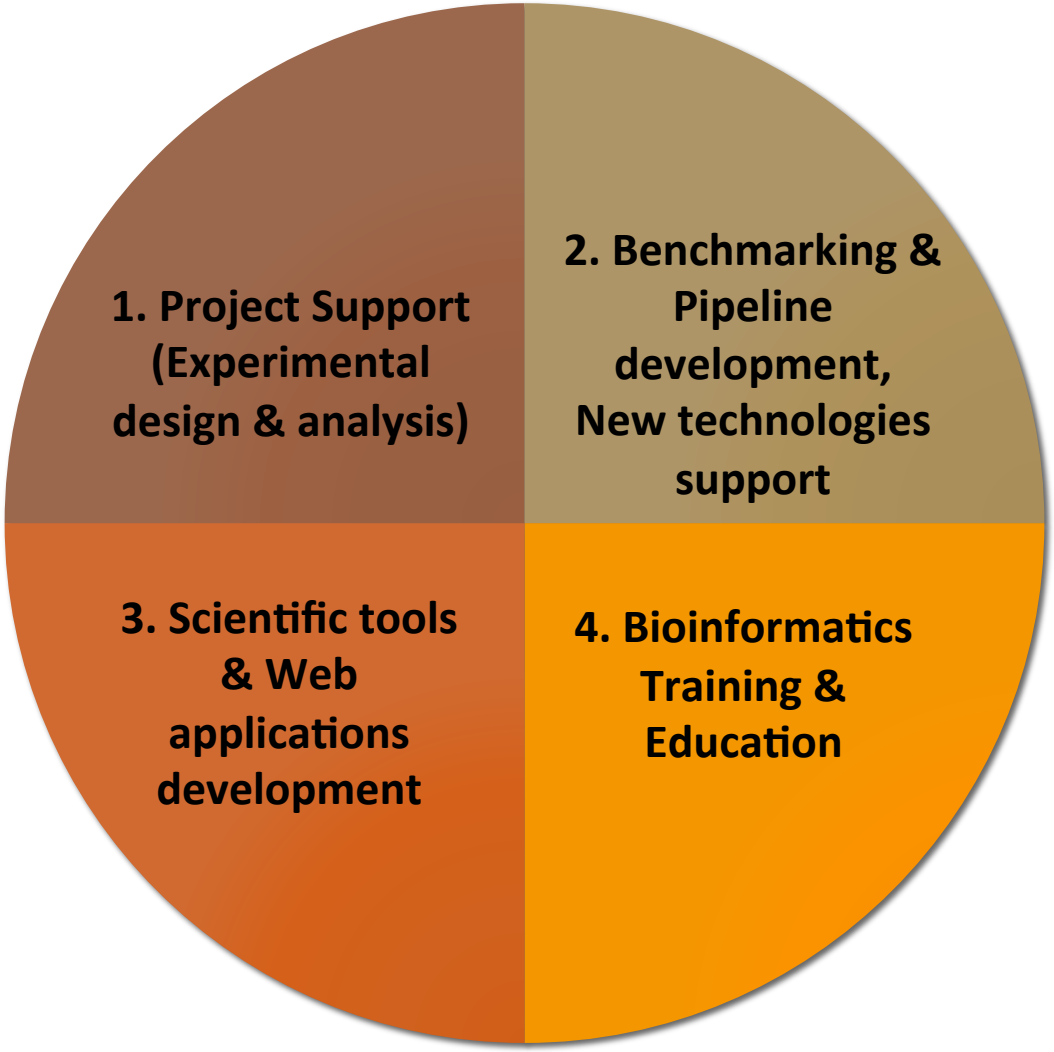
Vishal Koparde

Soon to be part of CDSL (CCR Cancer Data Science Lab)

Typical workflow



CCBR Functional Areas



1. Project Support

Current CCBR Projects

Total Number of	Total Number
Principal Investigators	95
Projects	189

Data Mining:

Analyzing public microarray data for Merkel cell carcinoma

Basic/Bench Research:

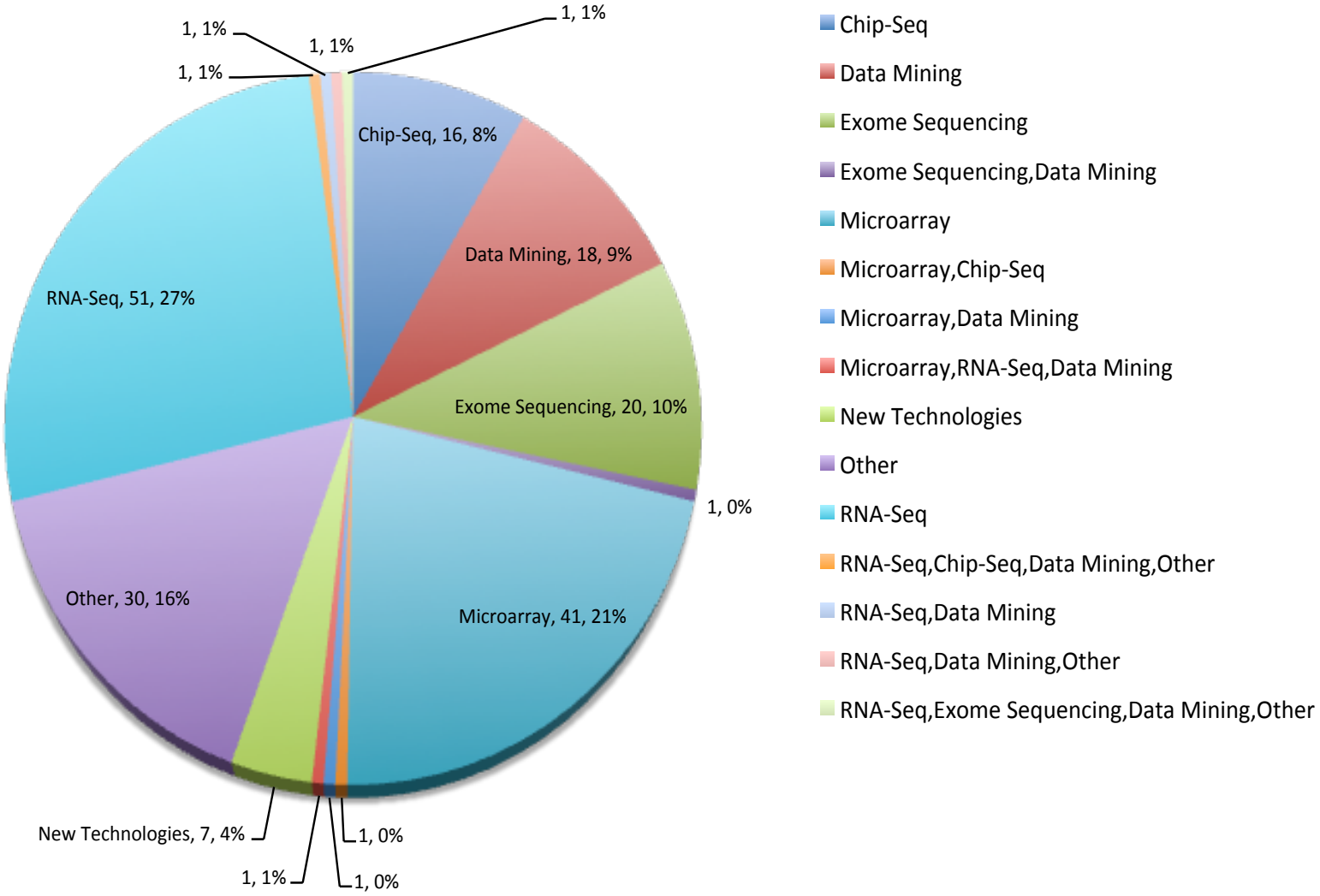
Study of alternative splicing function of Rbfox1 in knock out and transgenic mice
Disruption of Pol II Elongation with O-GlcNAcylation Inhibitors

Translational Research:

Genomic characterization of mouse model for GBM
Gene expression analysis of Kras-induced lung cancer mouse model

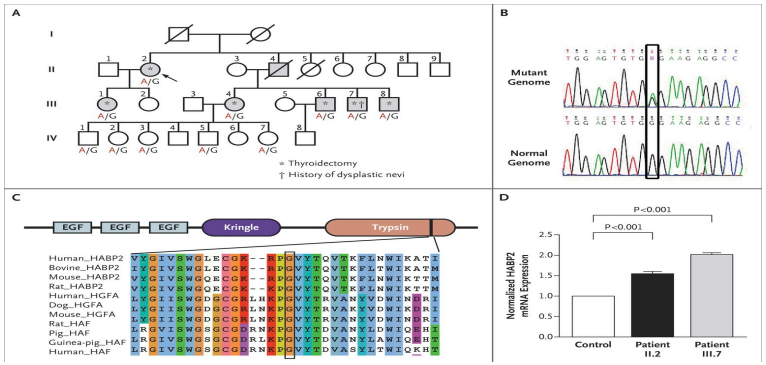
Clinical Research:

Germline Exome-Seq analysis of Familial Non-Medullary Thyroid Cancer
Exome-Seq analysis of adrenocortical cancer germ line and tumor DNA

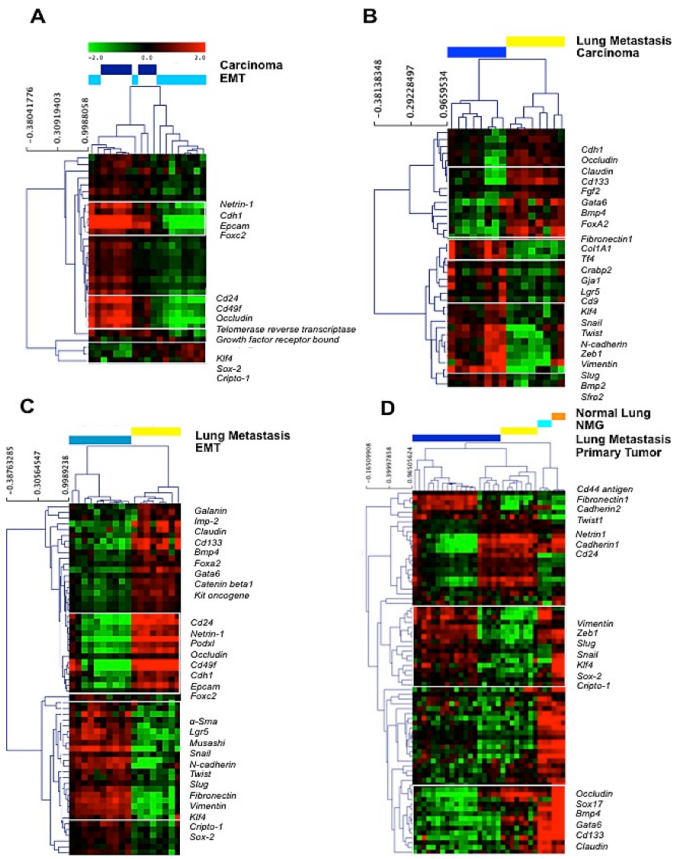

Breakdown of Project Types
Chip-Seq, 16, 8%
Data Mining, 18, 9%
Exome Sequencing, 20, 10%
1, 0%
Microarray, 41, 21%
1, 0%
1, 0%
1, 1%
New Technologies, 7, 4%
Other, 30, 16%
RNA-Seq, 51, 27%
1, 1%
1, 1%
1, 1%
Chip-Seq
Data Mining
Exome Sequencing
Exome Sequencing,Data Mining
Microarray
Microarray,Chip-Seq
Microarray,Data Mining
Microarray,RNA-Seq,Data Mining
New Technologies
Other
RNA-Seq
RNA-Seq,Chip-Seq,Data Mining,Other
RNA-Seq,Data Mining
RNA-Seq,Data Mining,Other
RNA-Seq,Exome Sequencing,Data Mining,Other

Some Projects

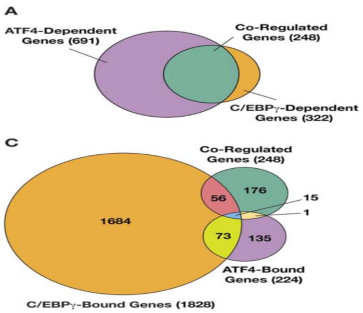
Germline mutations – Exome Seq Analysis



Gene Expression Analysis

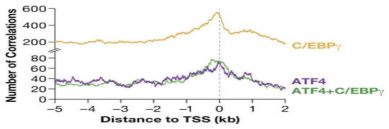


Integrated ChIP-Seq & Microarray Analysis



B

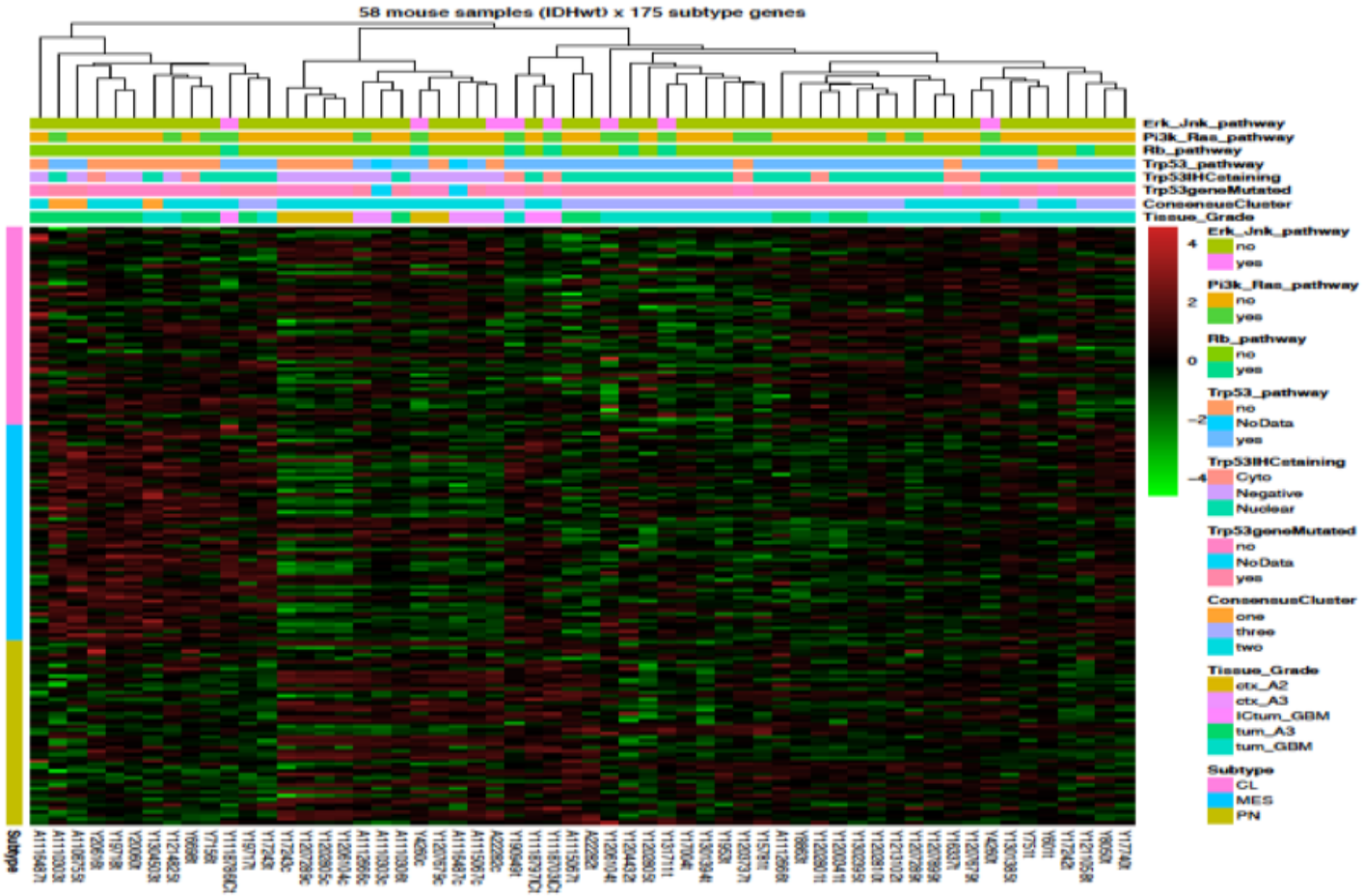
	Total Peaks	TSS Proximal Peaks	% TSS Proximal Peaks
ATF4	2642	233	9%
C/EBPγ	25419	2060	8%
ATF4+C/EBPγ	391	83	21%



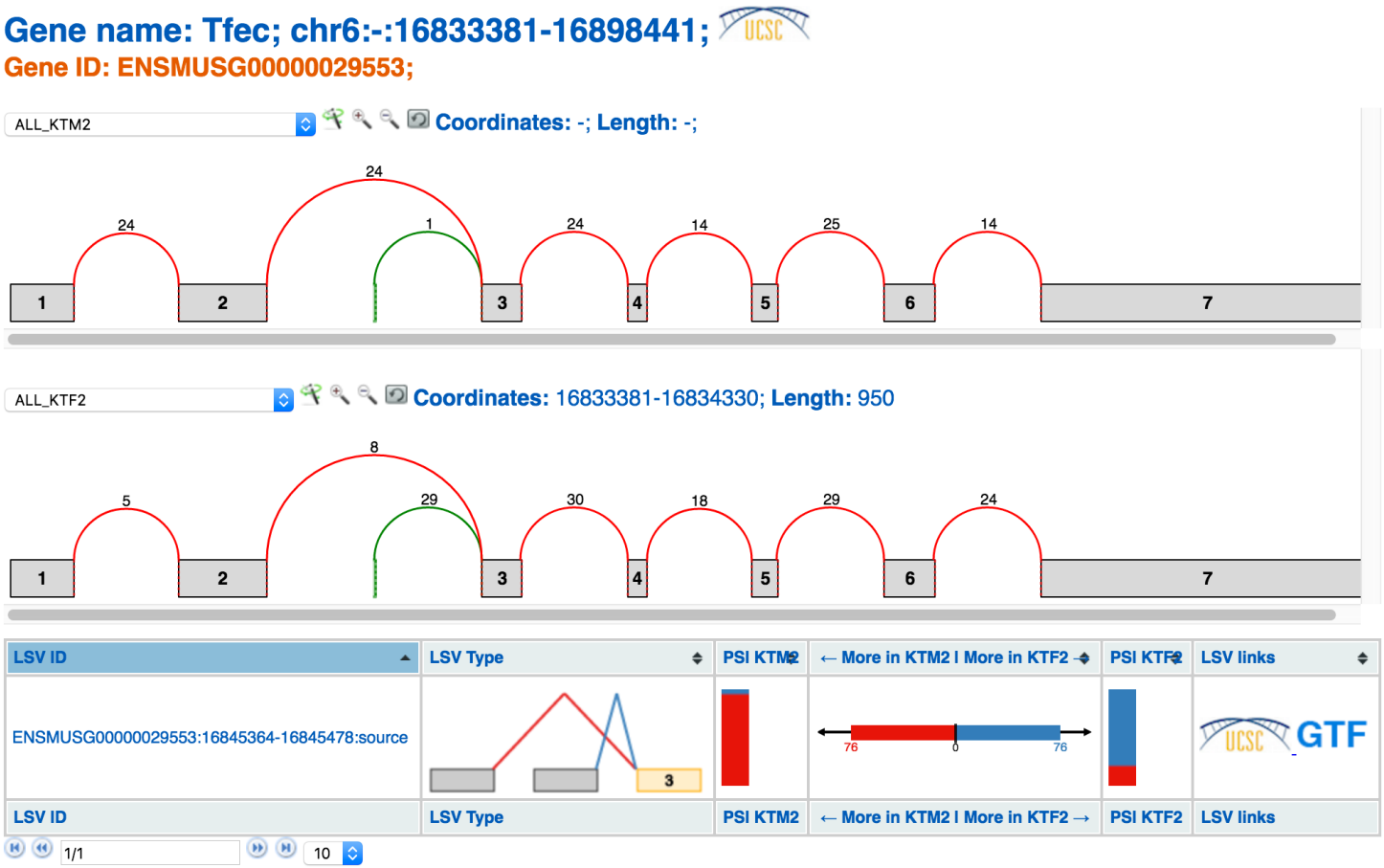
E

	Total Peaks	No. CARE-Containing Peaks (No. of Matches)	% CARE-Containing Peaks	No. TSS-Proximal Peaks	No. CARE-Containing TSS-Proximal Peaks (No. of Matches)	% TSS-Proximal CARE-Containing Peaks
ATF4	2642	391 (451)	14.7%	233	74 (88)	31.7%
C/EBPγ	25419	4033 (4486)	15.8%	2060	472 (526)	22.9%

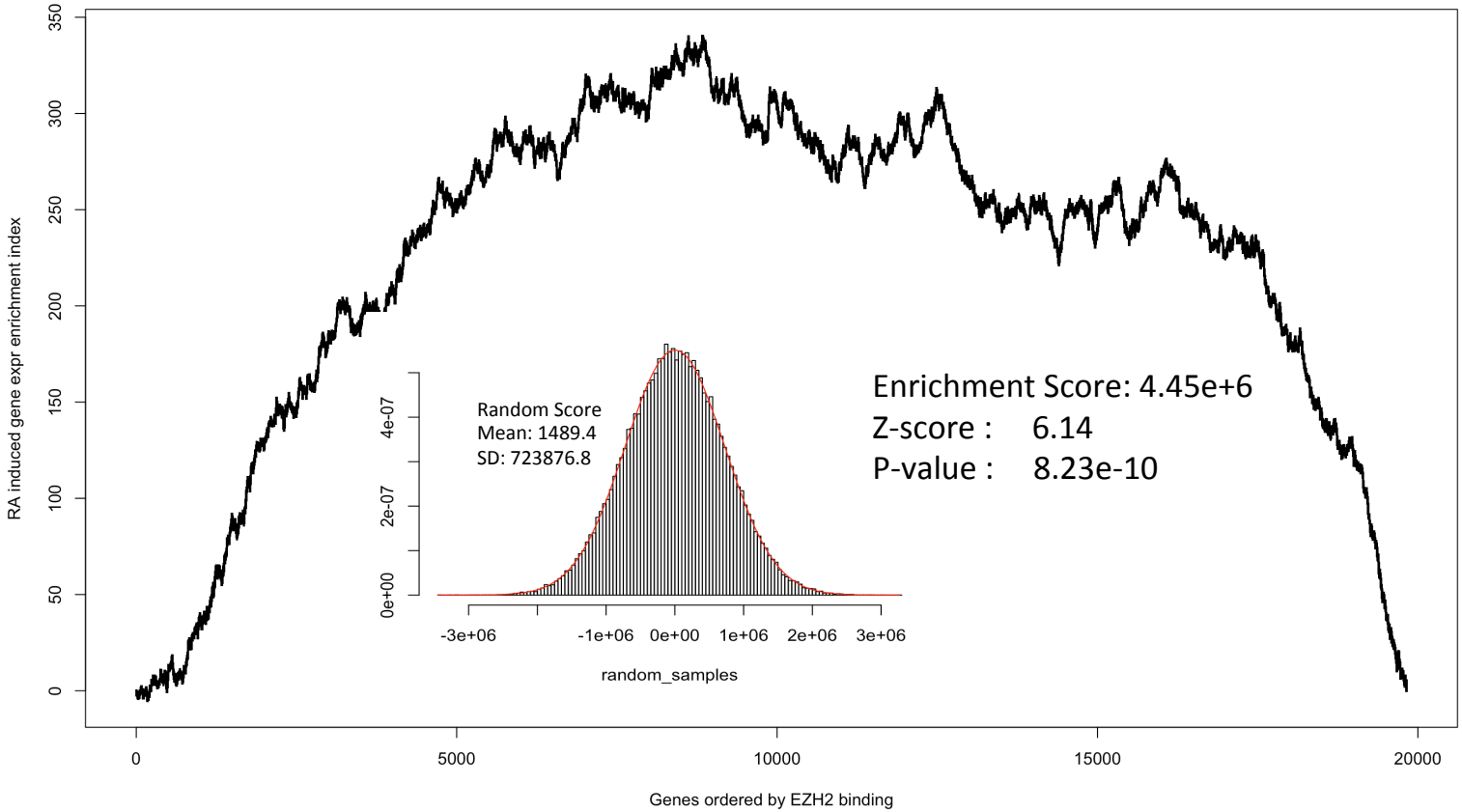
Classification of Mouse Glioma



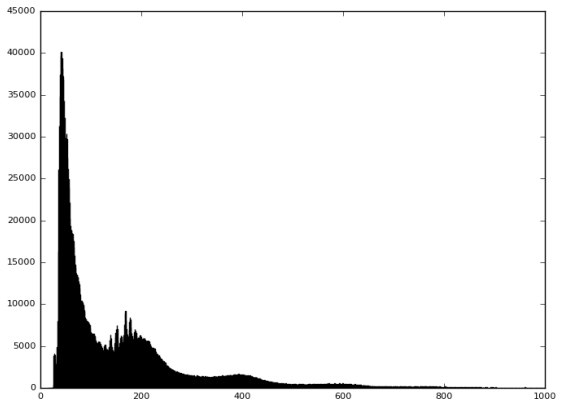
Splice Variant Analysis

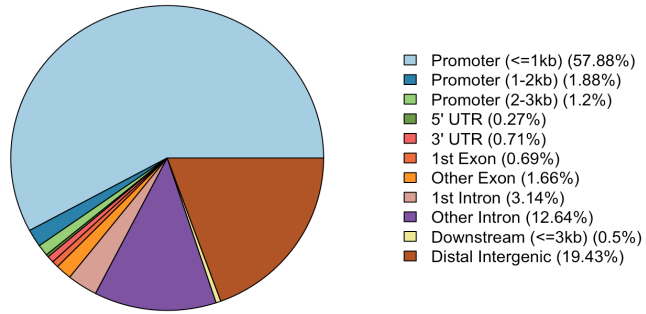


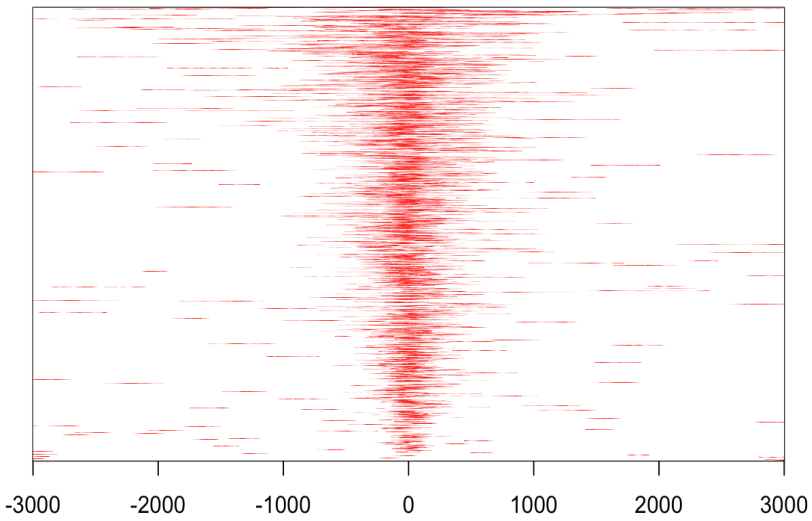
Integrated ChIP-Seq/RNA-Seq Analysis



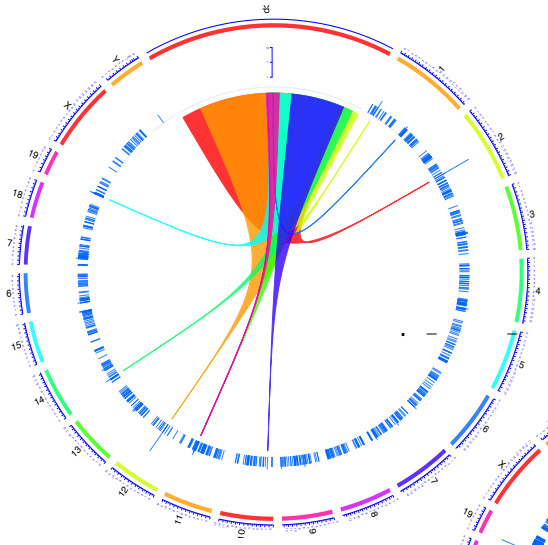
Chip-Seq/ATAC-Seq Project

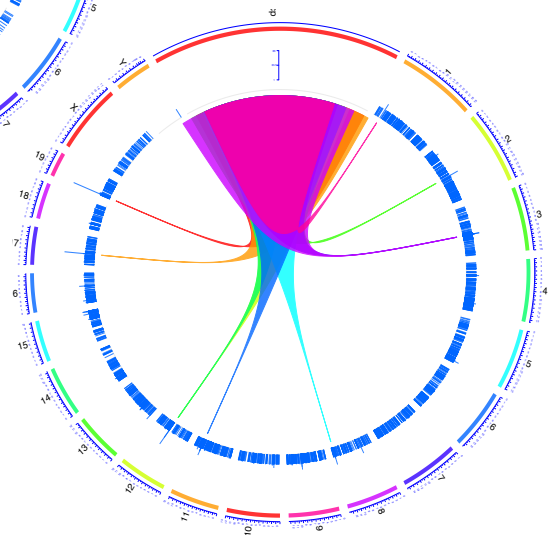


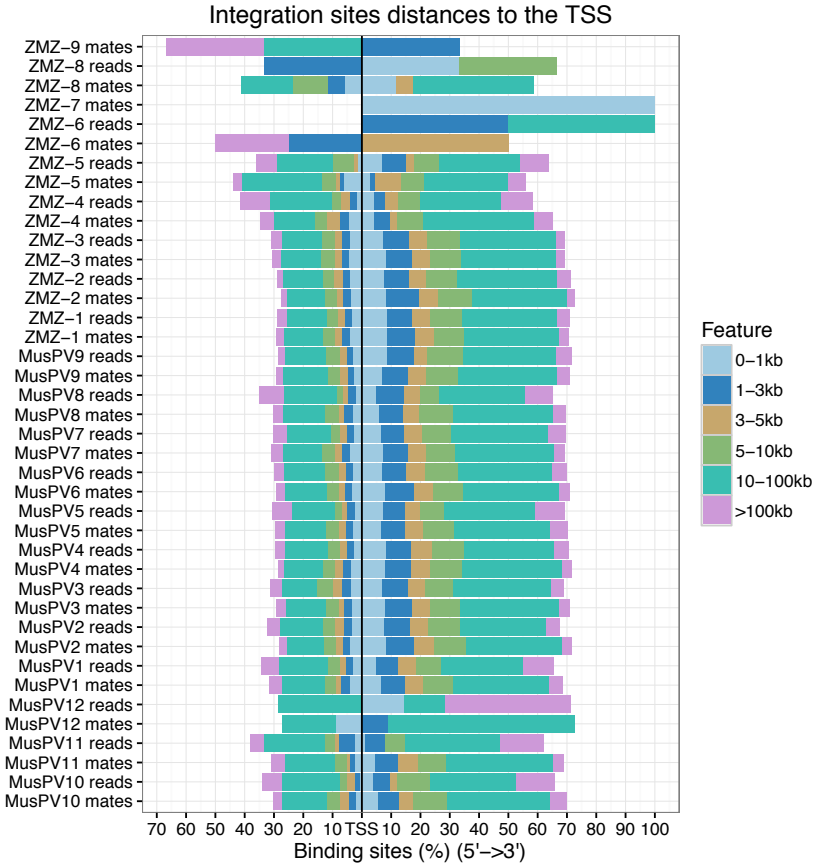




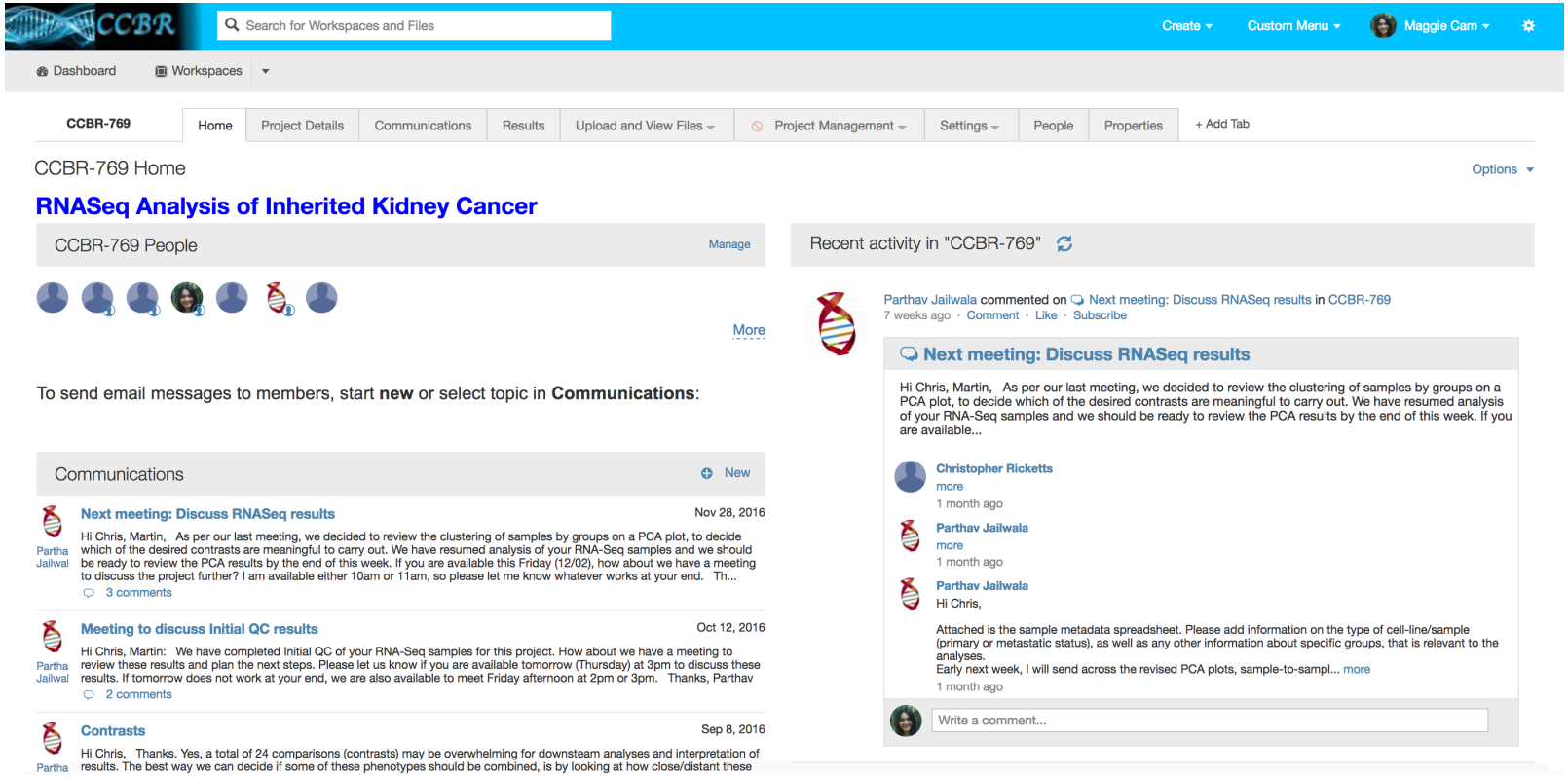
Virus Integration Analysis







Collaboration Website: iMeetCentral



CCBR Publications

Home » CCBR Publications

2016

- McCullen MV, Li H, **Cam M**, Sen S, McVicar DW, Anderson SK. Ly49 Pro1 element activity is associated with gene activation, not gene expression: Pro 1 does not function as an enhancer in the Ly49 genes and Pro1 transcripts are not present in mature Ly49-expressing NK cells. Genes and Immunity *advance online publication 28 July 2016; doi: 10.1038/gene.2016.31*
- Weyemi U, Redon CE, Sethi TK, Burrell AS, **Jailwala P**, **Kasoji M**, **Abrams N**, **Merchant A**, Bonner WM. Twist1 and Slug mediate H2AX-regulated Epithelial-Mesenchymal Transition in breast cells. Cell Cycle. 2016 *Jun 17:0. [Epub ahead of print]*
- Matter MS, Marquardt JU, Andersen JB, Quintavalle C, Korokhov N, Stauffer JK, Kaji K, Decaens T, Quagliata L, **Elloumi F**, Hoang T, Molinolo A, Conner EA, Weber A, Heikenwalder M, Factor VM, Thorgeirsson SS. Oncogenic driver genes and the inflammatory microenvironment dictate liver tumor phenotype. Hepatology. *2016 Jun;63(6):1888-99. doi: 10.1002/hep.28487. Epub 2016 Mar 15.*
- Mendoza-Villanueva D, Balamurugan K, Ali HR, Kim SR, Sharan S, Johnson RC, **Merchant AS**, Caldas C, Landberg G, Sterneck E. The C/EBPδ protein is stabilized by estrogen receptor α activity, inhibits SNAI2 expression and associates with good prognosis in breast cancer. Oncogene. *2016 May 16. doi: 10.1038/onc.2016.156. [Epub ahead of print].*
- Bae HR, Leung PS, Tsuneyama K, Valencia JC, Hodge DL, Kim S, Back T, Karwan M, **Merchant AS**, Baba N, Feng D, Park O, Gao B, Yang GX, Eric Gershwin M, Young HA. Chronic Expression of Interferon Gamma Leads to Murine Autoimmune Cholangitis with a Female Predominance. Hepatology. *2016 May 14. doi: 10.1002/hep.28641. [Epub ahead of print].*
- Rothermel LD, Sabesan AC, Stephens DJ, Chandran SS, Paria BC, Srivastava AK, Somerville R, Wunderlich JR, Lee CC, Xi L, Pham TH, Raffeld M, **Jailwala P**, **Kasoji M**, Kammula US. Identification of an Immunogenic Subset of Metastatic Uveal Melanoma. Clin Cancer Res. 22:2237-49, 2016.
- Kennedy MW, Chalamalasetty RB, Thomas S, Garriock RJ, **Jailwala P**, Yamaguchi TP. Sp5 and Sp8 recruit β-catenin and Tcf1-Lef1 to select enhancers to activate Wnt target gene transcription. Proc Natl Acad Sci U S A. 113:3545-50, 2016.
- Weyemi U, Redon CE, Choudhuri R, Aziz T, Maeda D, Boufraqech M, Parekh PR, Sethi TK, **Kasoji M**, **Abrams N**, **Merchant A**, Rajapakse VN, Bonner WM. The histone variant H2A.X is a regulator of the epithelial-mesenchymal transition. Nat Commun 7:10711, 2016.
- Dine JL, O'Sullivan CC, Voeller D, Greer YE, Chavez KJ, Conway CM, Sinclair S, Stone B, Amiri-Kordestani L, **Merchant AS**, Hewitt SM, Steinberg SM, Swain SM, Lipkowitz S. The TRAIL receptor agonist drozitumab targets basal B triple-negative breast cancer cells that express vimentin and Axl. Breast Cancer Res Treat. 155:235-51, 2016.
- Castro NP, **Merchant AS**, Saylor KL, Anver MR, Salomon DS, Golubeva YG. Adaptation of Laser Microdissection Technique for the Study of a Spontaneous Metastatic Mammary Carcinoma Mouse Model by NanoString Technologies. PLoS One. 11:e0153270, 2016.

How to get started

Contact maggie.cam@nih.gov or CCBR@mail.nih.gov

Drop by: Bldg 37, Rm 3041 (office hours 10-12am)

For significant help, trigger project request: Bioinformatics.cancer.gov ("Ask for Help")

Appointment: Discuss experimental design, analysis, goals and timelines

After data arrives: analyst is assigned to project and additional meeting(s) for further discussion

2. Research & Development

R&D

Joint effort of ATRF Sequencing Facility Bioinformatics Team and CCBR

Benchmarking of current and new algorithms for routine use

- Exome-seq, RNA-Seq, ChIP-Seq, miR-Seq

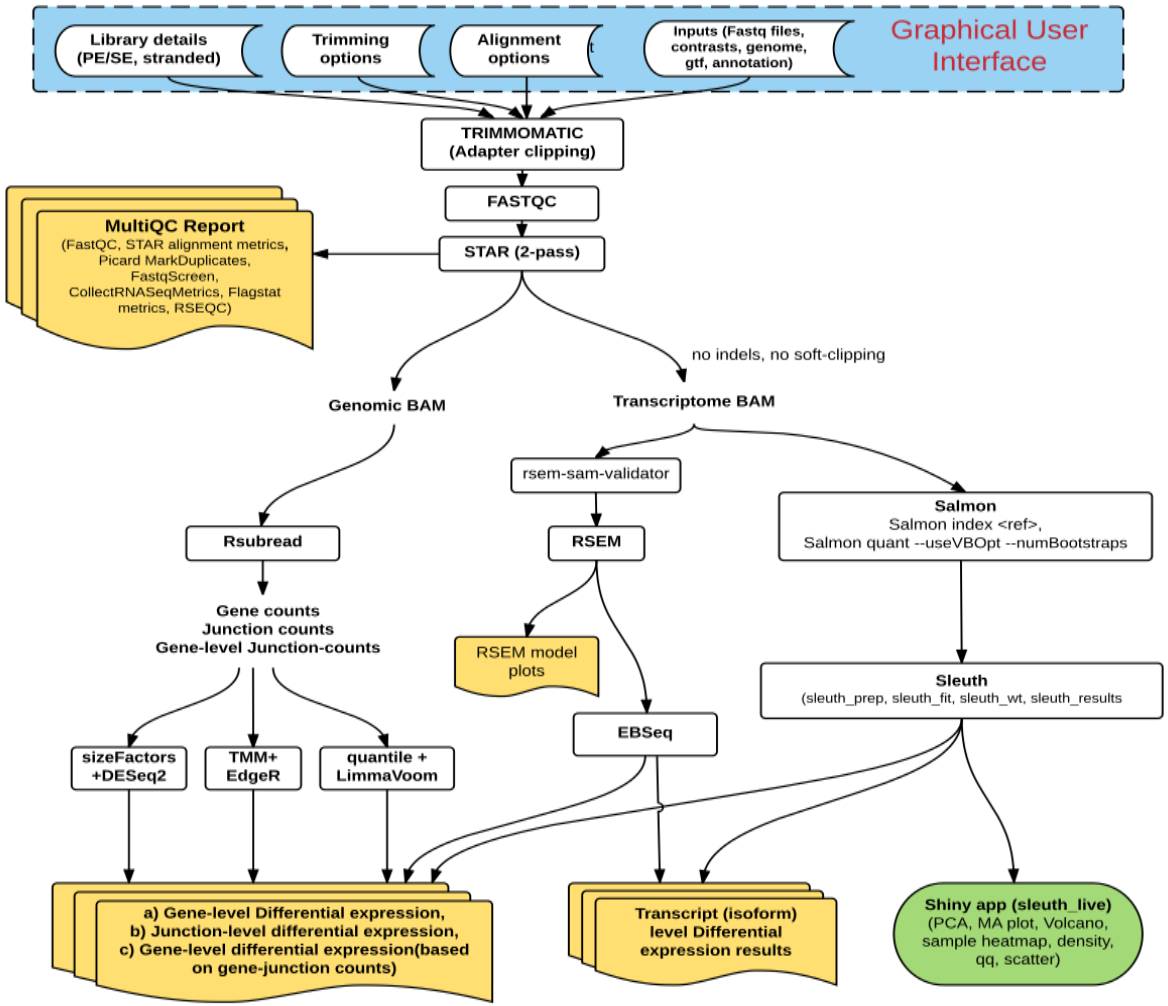
Pipeline development

- Methods for standard workflows selected based on benchmarking
- Streamline upstream QC methods from ATRF and downstream analysis
- Standardize methods for reproducibility, updated versions as needed
- Publish in GitHub for CCR/NIH, also useful for others

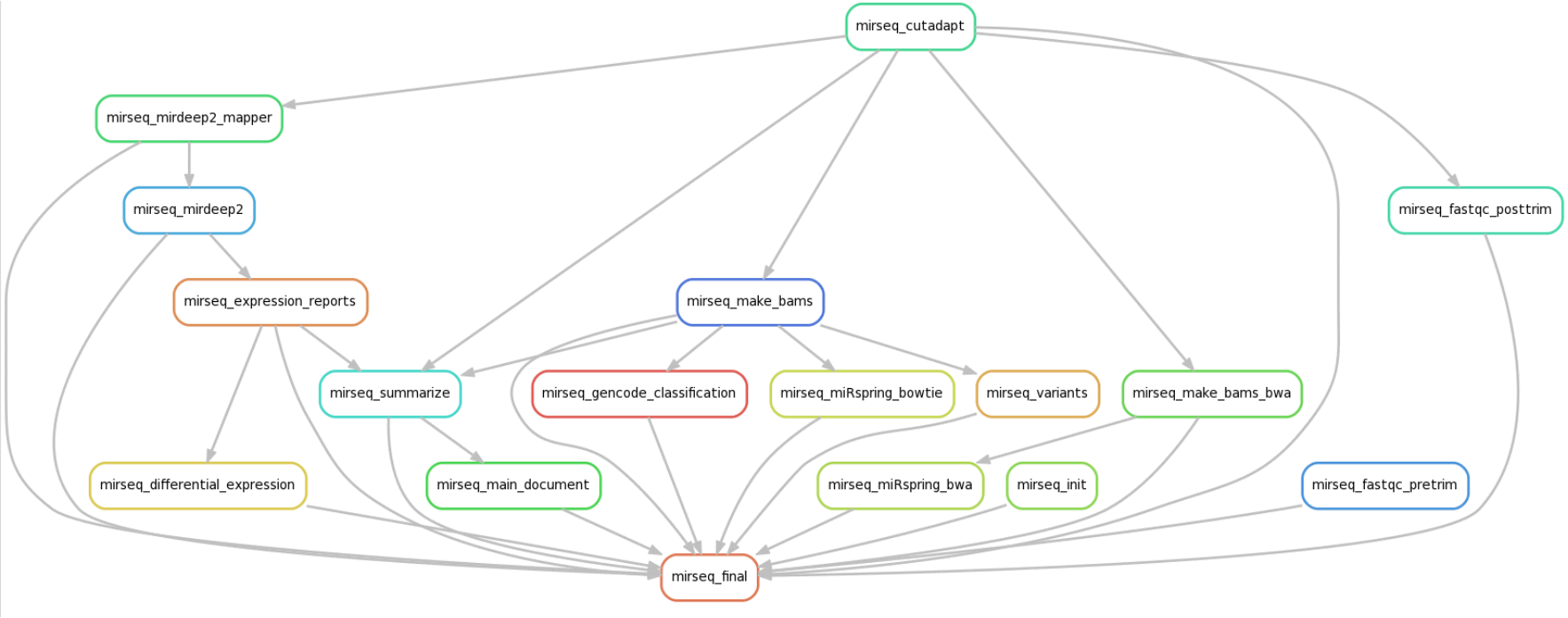
Additional tool development

- Novel tools and algorithms arising from collaborative projects
- Published in GitHub and/or made available through web application

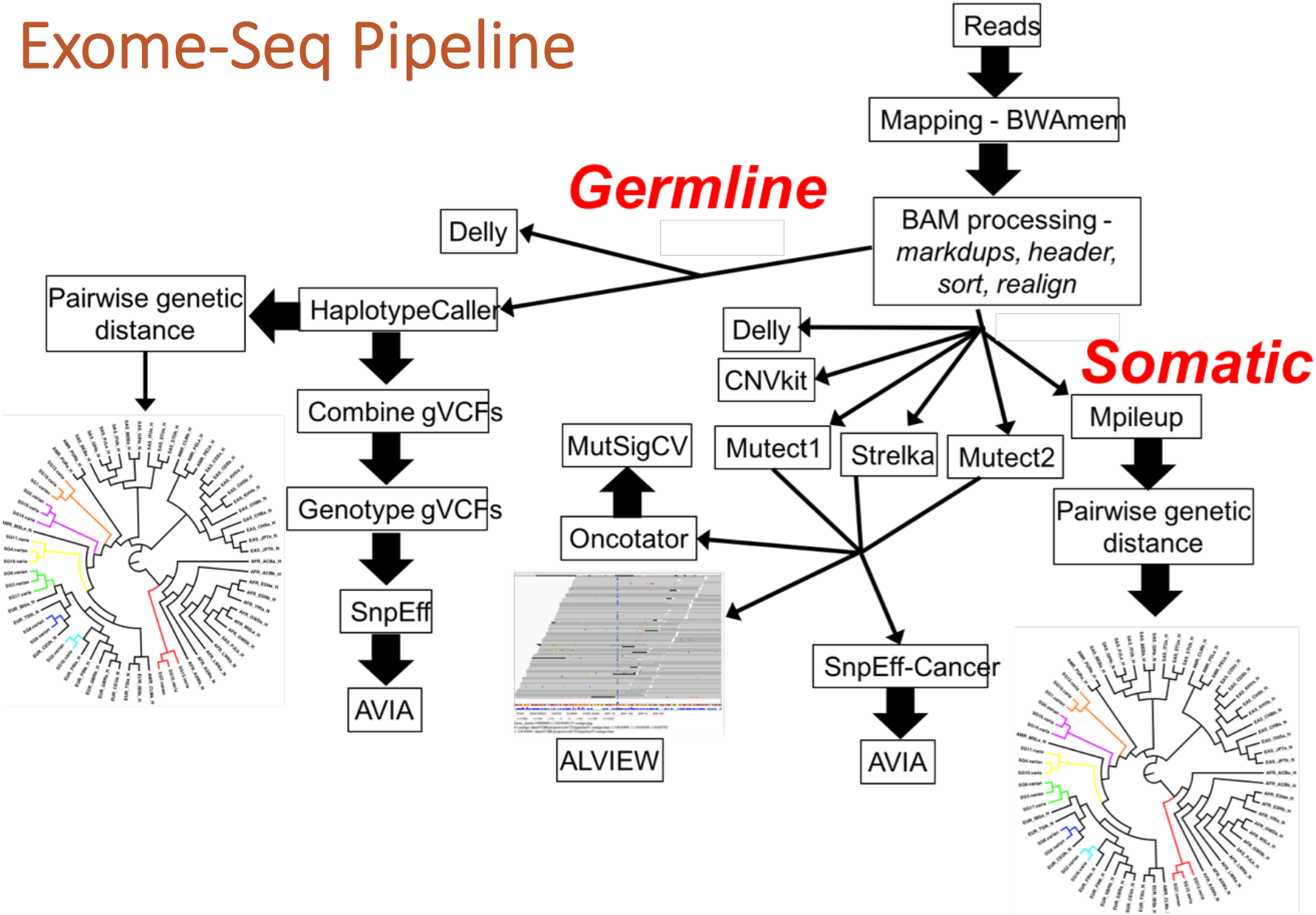
RNA-Seq Pipeline



miR-Seq Pipeline (coming soon)



Exome-Seq Pipeline

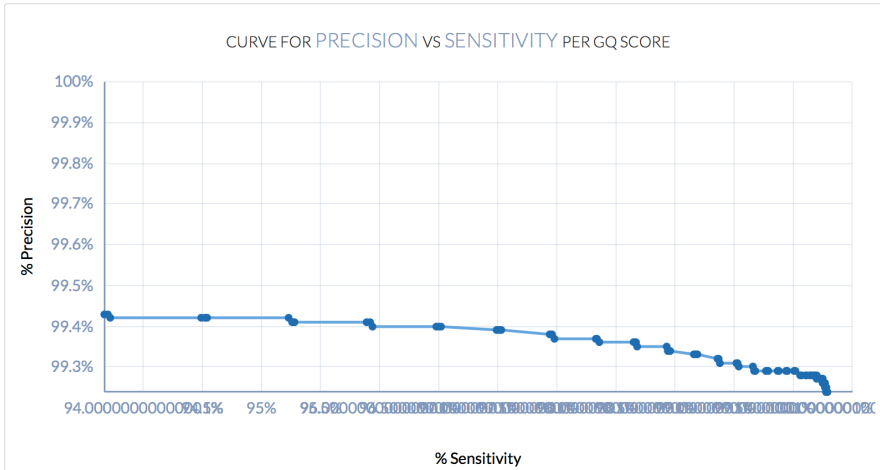


Precision FDA Challenge

Old, GRCh37 reference genome

PRECISION	99.24%
RECALL	99.79%
F-MEASURE	99.51%

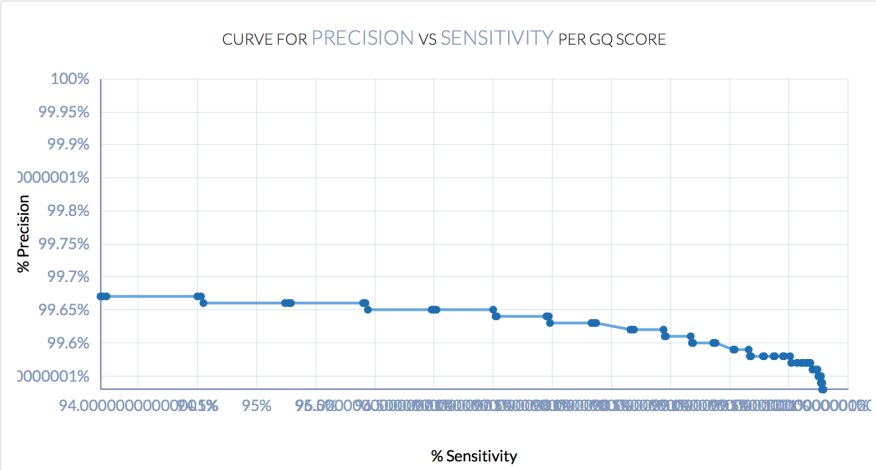
TRUE-POSITIVES	3,144,270
FALSE-POSITIVES	24,156
FALSE-NEGATIVES	6,749



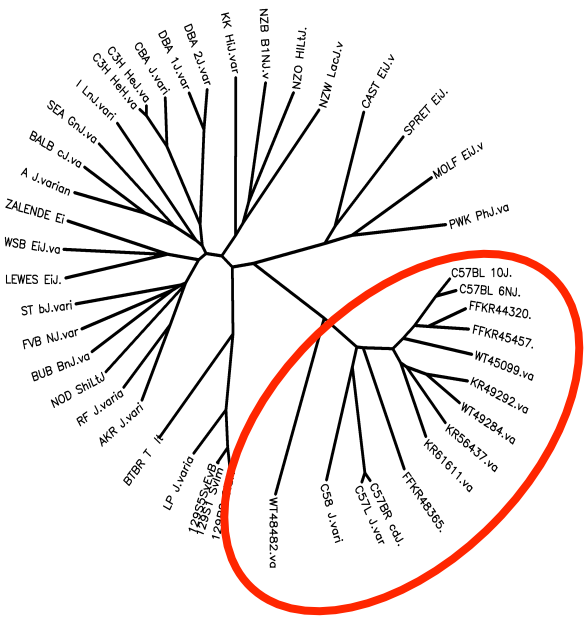
New, hs37d5 reference genome

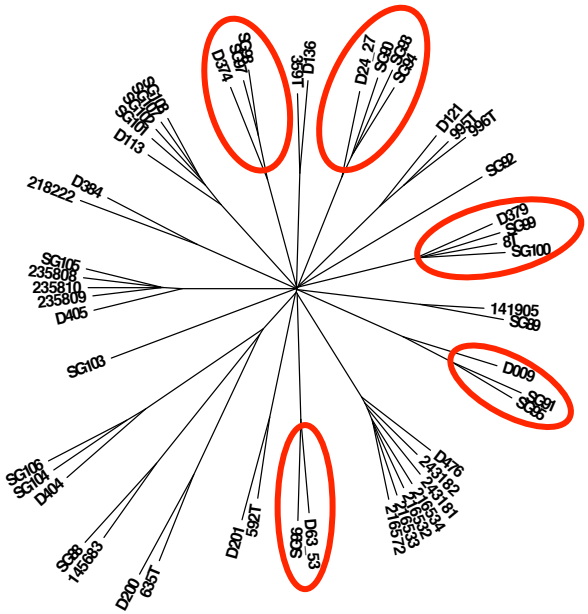
PRECISION	99.53%
RECALL	99.79%
F-MEASURE	99.66%

TRUE-POSITIVES	3,144,362
FALSE-POSITIVES	14,933
FALSE-NEGATIVES	6,657

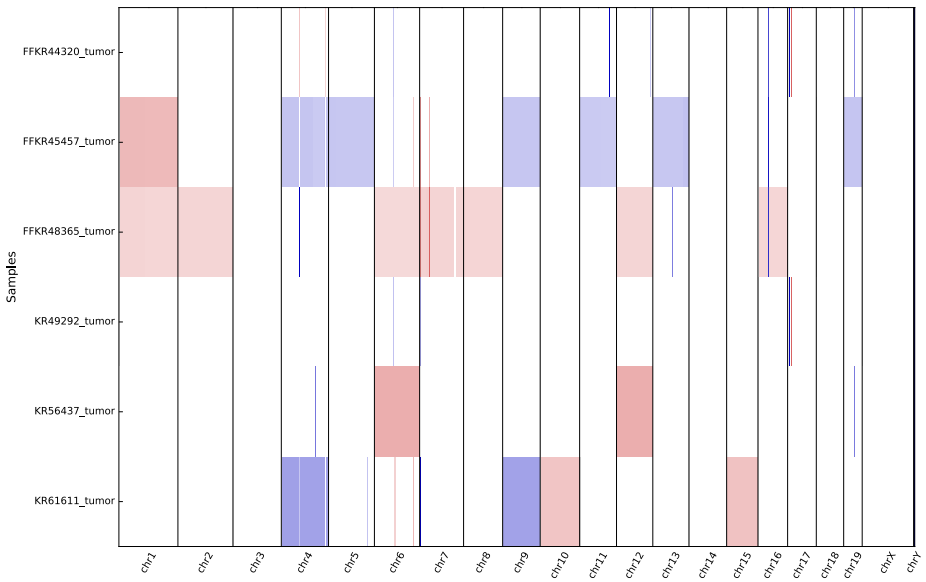


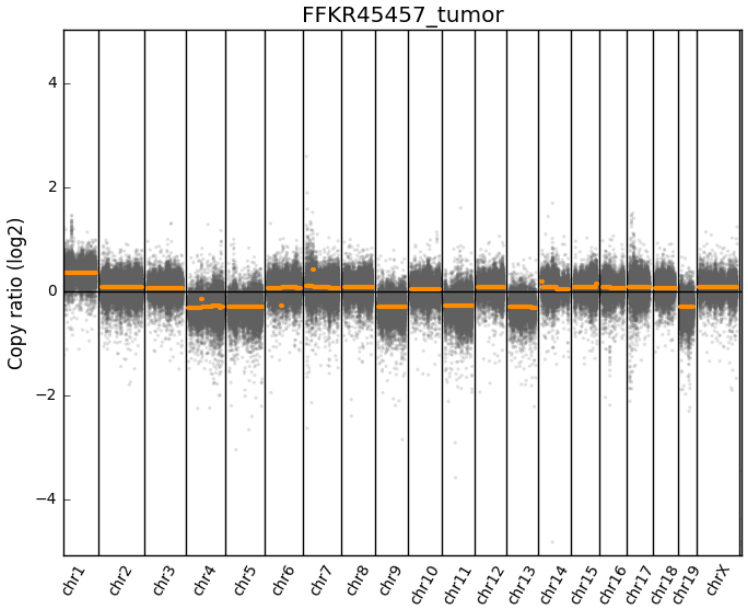
QC – Sample Identification



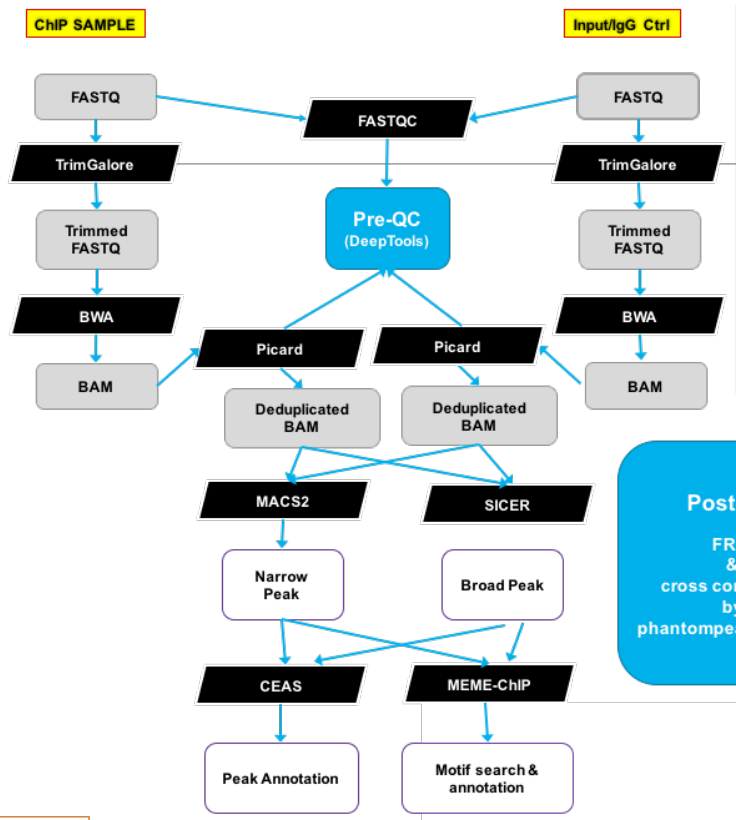


CNVkit – amplification/deletion detection





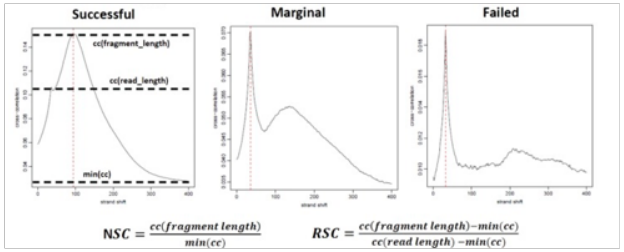
ChIP-Seq Pipeline (coming soon)



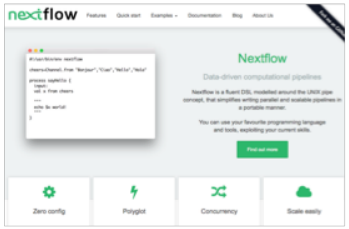

```
[kimb8@biowulf ChIP-Seq-Pipeline]$
[kimb8@biowulf ChIP-Seq-Pipeline]$ nextflow run main.nf --reads='example/*.fastq' --macsconfig=
'example/macssetup.config' -with-timeline timeline.html -with-dag dag.png
N E X T F L O W  ~  version 0.21.3
Launching main.nf
[warm up] executor > slurm
[a0/b76b27] Submitted process > fastqc (polii_1d_co.fastq)
[10/5f3575] Submitted process > fastqc (input_2d_ra.fastq)
[e5/551f45] Submitted process > fastqc (mycn_2d_co.fastq)
[9d/1eeba2] Submitted process > fastqc (polii_1d_ra.fastq)
[3a/94cc96] Submitted process > fastqc (input_1d_co.fastq)
[25/ddcfe3] Submitted process > fastqc (h3k4me3_1d_ra.fastq)
[c7/201647] Submitted process > fastqc (input_1d_ra.fastq)
[30/04cd5f] Submitted process > fastqc (mycn_2d_ra.fastq)
[a1/576ac9] Submitted process > fastqc (input_2d_co.fastq)
[33/2b9801] Submitted process > fastqc (h3k4me3_1d_co.fastq)
[39/daa8e4] Submitted process > trimgalore (polii_1d_co.fastq)
```

Post-QC

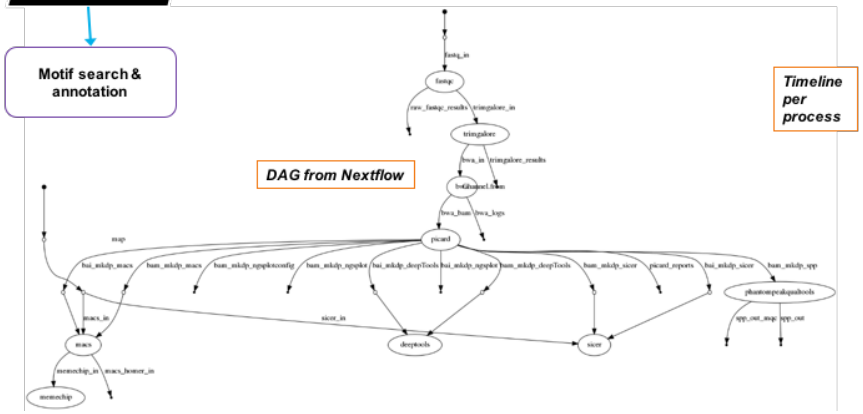
FRiP
&
cross correlation
by
phantompeakqualtools



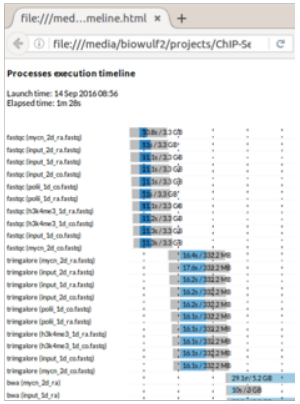
Powered by Nextflow



DAG from Nextflow



Timeline per process



Microarray Analysis App (coming soon)

CCBR Microarray analysis workflow

(For Affymetrix human and mouse data)

Project ID:

ccbr

Select CEL files

Choose Files no files selected

Choose phenotype file

Choose File no file selected

Choose contrast file

Choose File no file selected

KEGG/GO Enrichment Pvalue threshold

0.05

Which contrast to show

1

KEGG/GO Enrichment logFC threshold

1.5

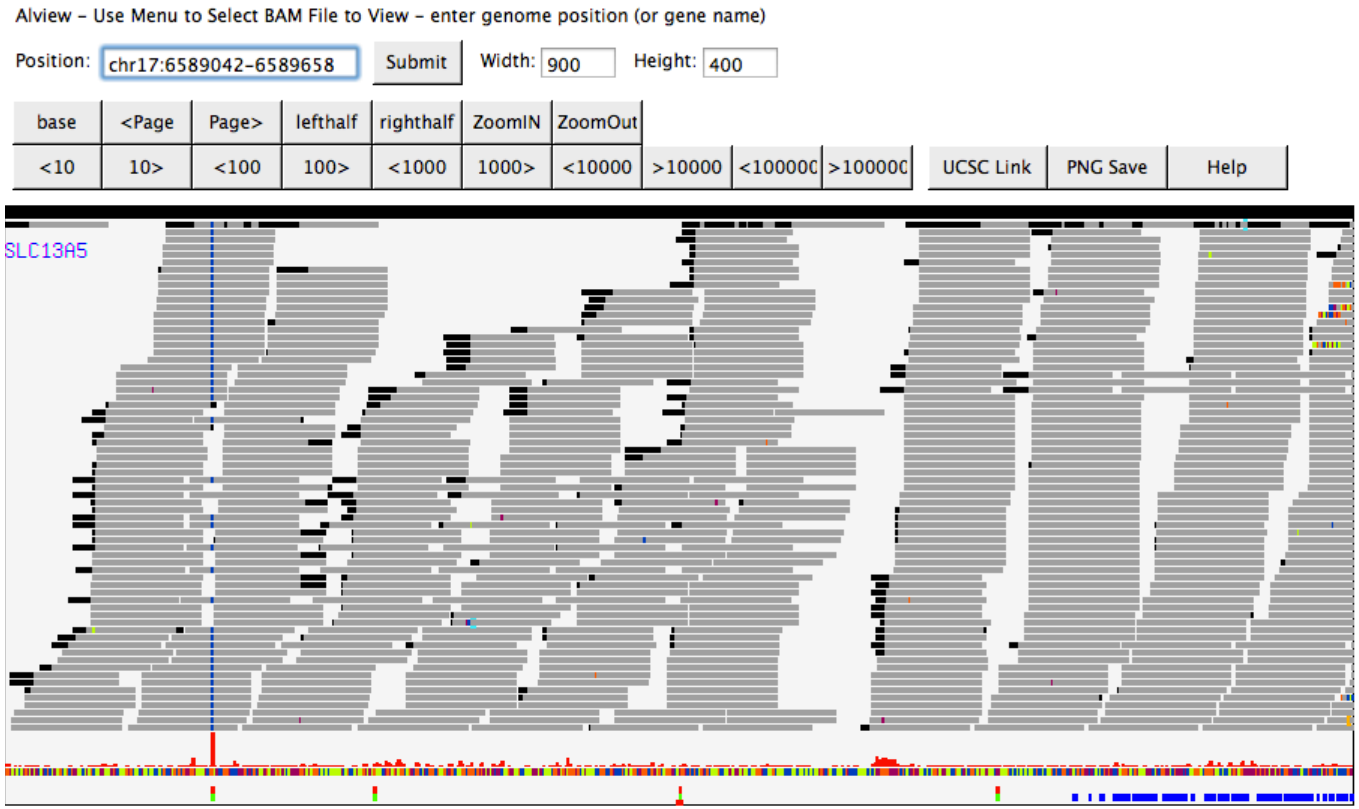
Start | Generate Report | Download Report | Download Tables

Microarray | Results | Pre-normalization QC plots | Post-normalization plots | DEG-Enrichments-tables | Help

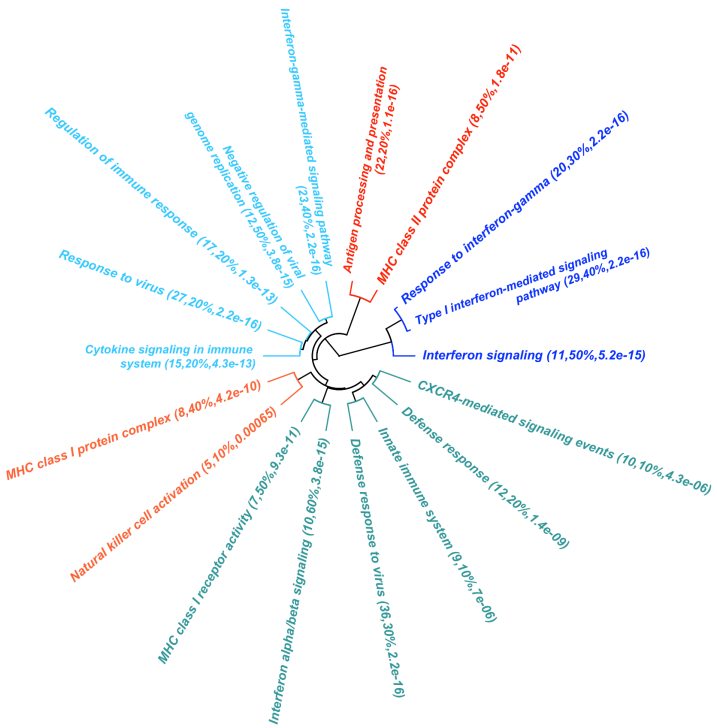
- Histogram
- Maplots
- Boxplots
- RLE
- NUSE

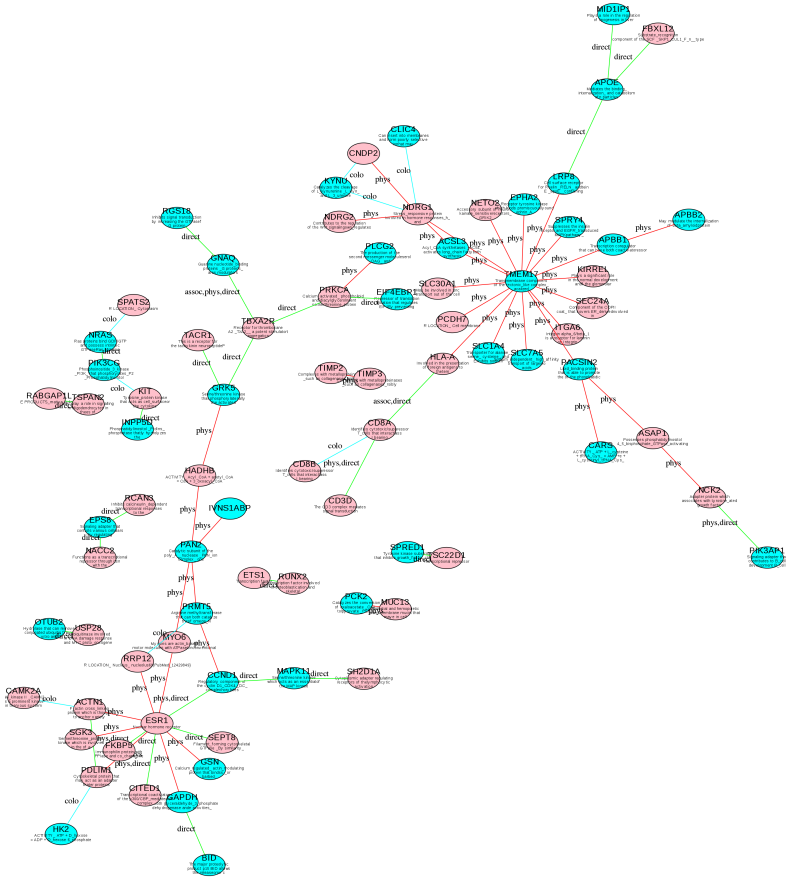
3. Tool and web applications development

Alview: bam file viewer

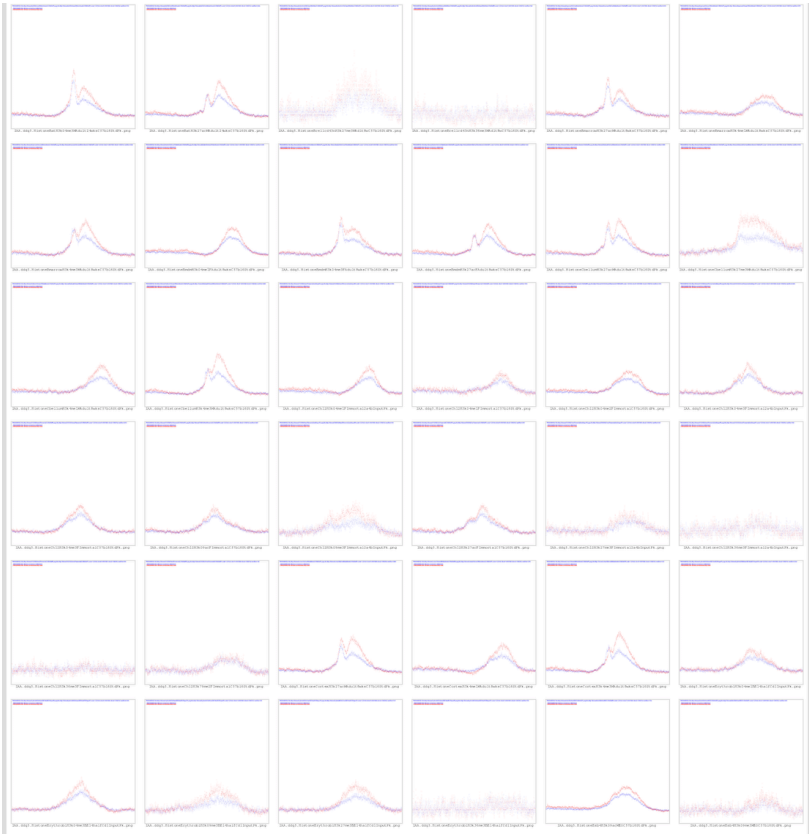


Pathway Analysis Tool: Pathway Enrichment/Network

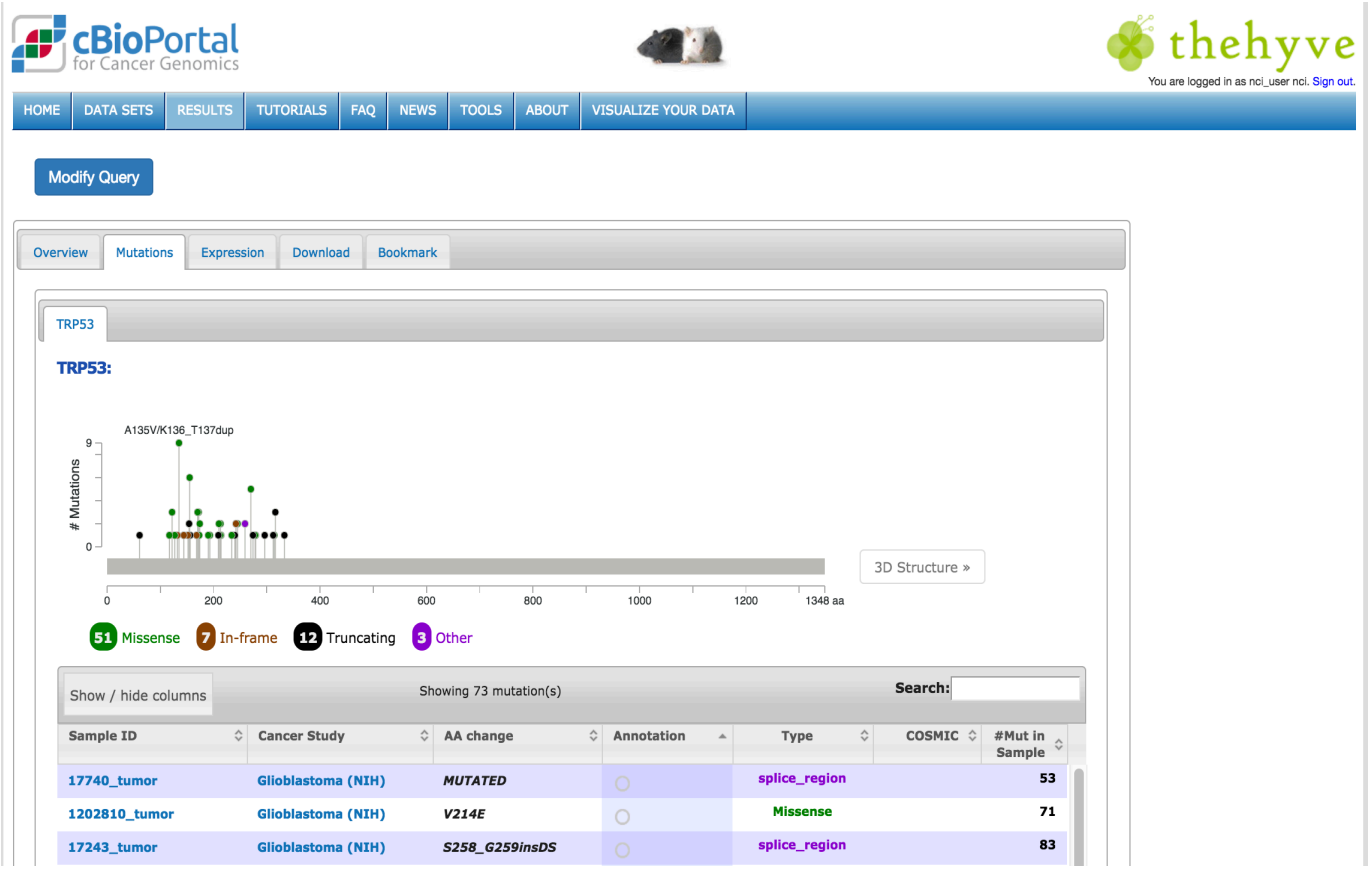




Pathway Analysis Tool: Encode Miner



cBioPortal for Mouse Cancer Models



4. Bioinformatics Training

BTEP Classes

- Microarray
- Best Practices/Experimental Design
- Exome-Seq
- RNA-Seq
- Chip-Seq

Training on CCBR Pipeliner

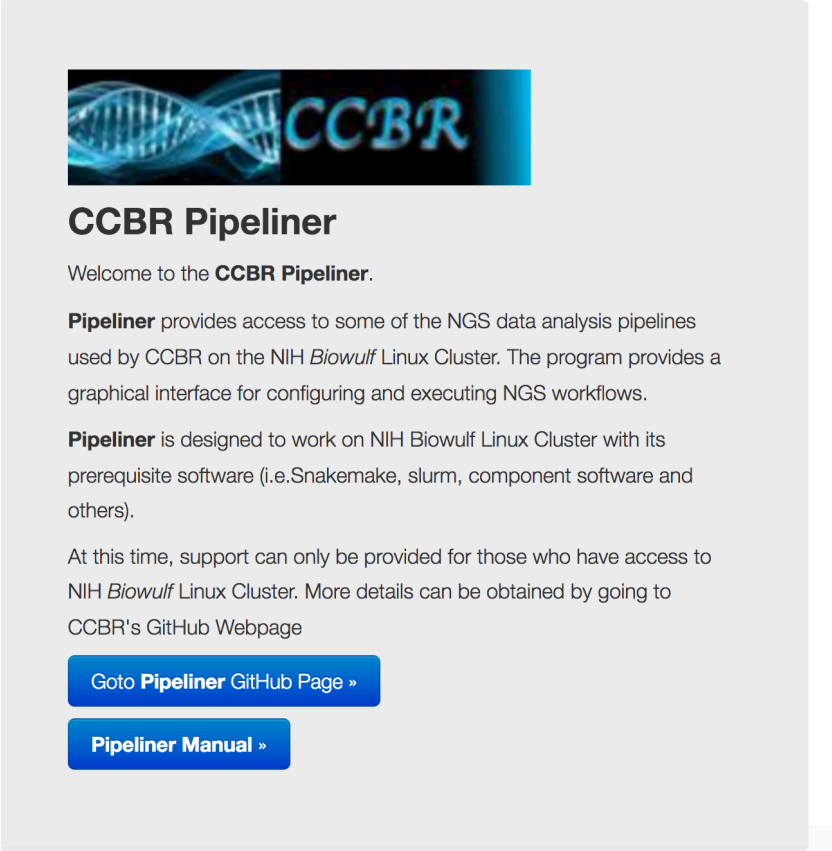
Small groups (max 6-7 people)

Biowulf account

Some command line knowledge

https://ccbr.github.io/Pipeliner/

mail: ccbr@mail.nih.gov



Some NGS Best Practices

RNA-Seq

Exome-Seq: Justin Lack

Chip-Seq: Bong-Hyun Kim

Intro and Best Practices: RNA-Seq

RNA-Seq Applications

Differential Gene Expression

- Looks at genes that are at least at the detection limit of microarrays
- Most straightforward, requires less read depth (10-30 M reads)
- Can be more cost-effective than microarrays

Differential Transcript Expression (Isoform switching)

- Still confined to known transcripts / isoforms
- Complexity is in the assignment of exons to particular isoforms
- Many algorithms can differ in results

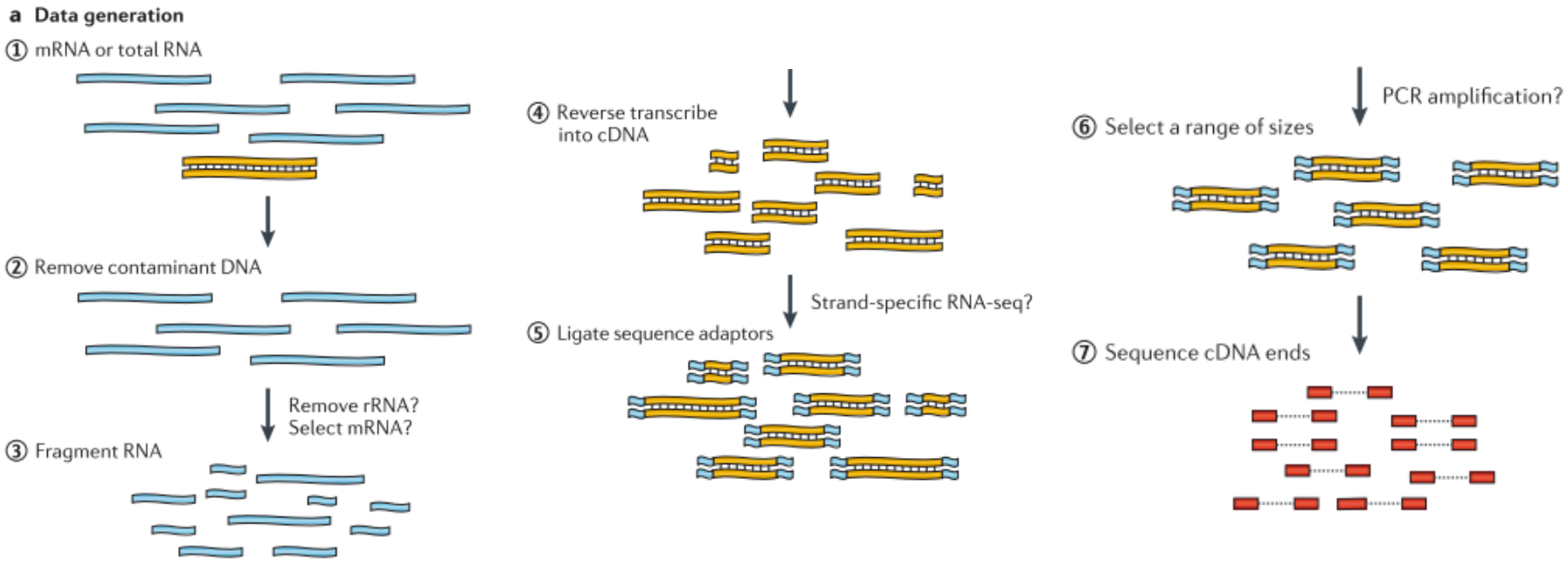
Transcript Discovery / Whole Transcriptome Profiling

- Interest is in looking for new isoforms or unannotated genes
- More complex in terms of bioinformatics analysis
- Can find false positives, depending on leniency of algorithm

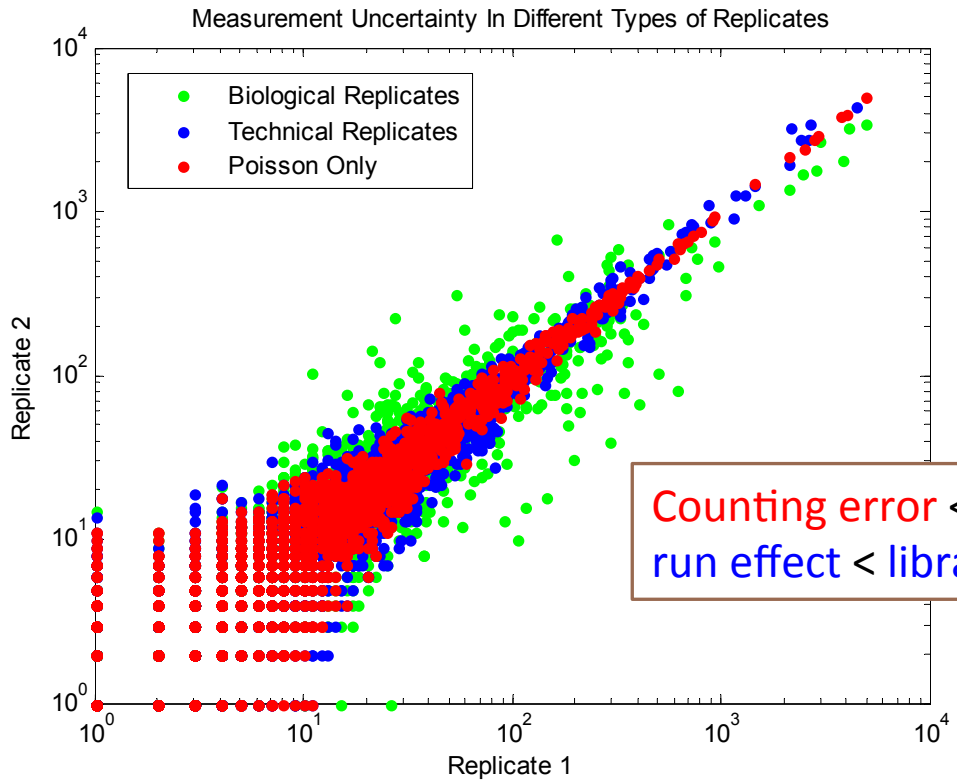
Others

- SNP/Somatic Variant/Gene Fusion Detection

Method – Preparation



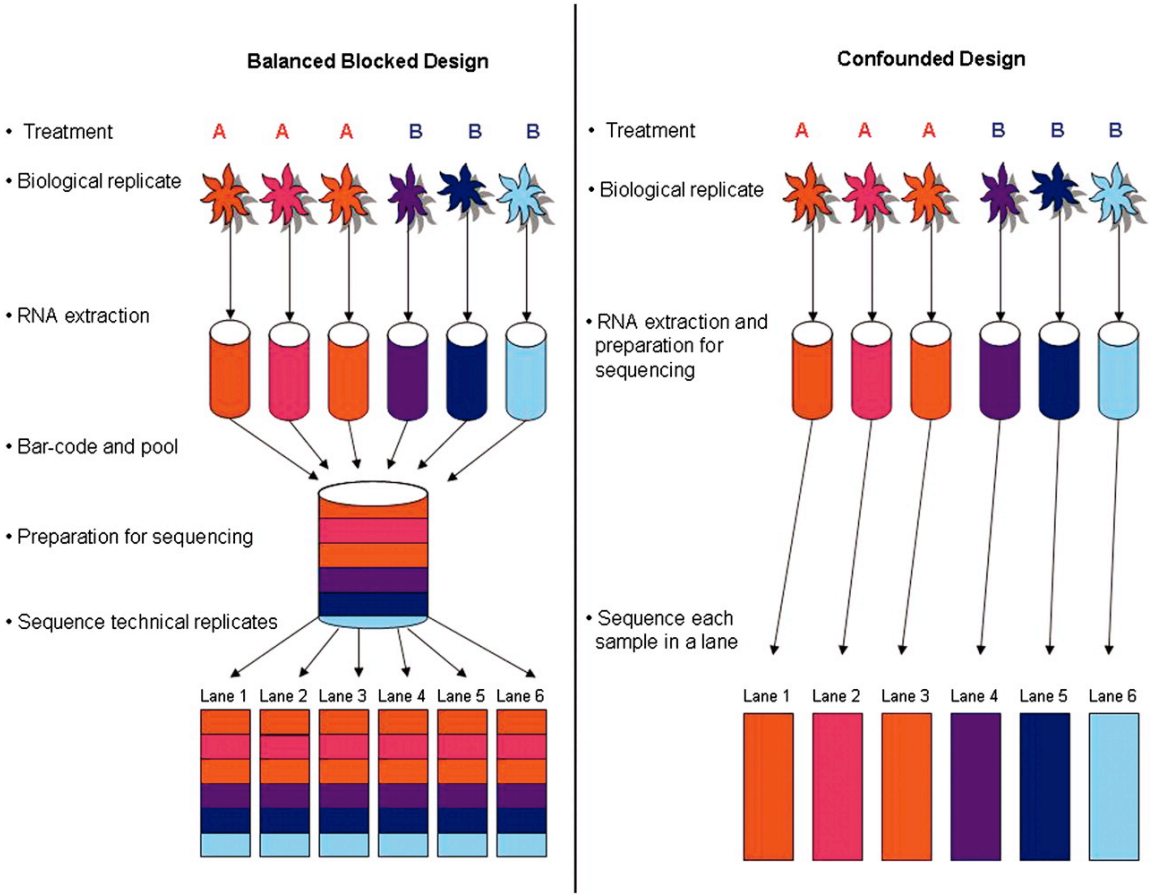
Types of variance



Counting error < Technical effects (lane effect < run effect < library prep effect) << biological effect

Busby et al, Bioinformatics 2013
Marioni et al, Genome Res 2008

Experimental Design: avoiding lane effects



- does not permit partitioning of batch and lane effects from the estimate of within-group biological variability

Auer and Doerge, Genetics 2010

Best Practices

1. Factor in at least 3 replicates (absolute minimum), but 4 if possible (optimum minimum). Biological replicates are recommended rather than technical replicates.

2. Always process your RNA extractions at the same time. Extractions done at different times lead to unwanted batch effects.

3. There are 2 major considerations for RNA-Seq libraries:

If you are interested in coding mRNA, you can select to use the mRNA library prep. The recommended sequencing depth is between 10-20M paired-end (PE) reads. Your RNA has to be high quality (RIN > 8).

If you are interested in long noncoding RNA as well, you can select the total RNA method, with sequencing depth ~25-60M PE reads. This is also an option if your RNA is degraded.

4. Ideally to avoid lane batch effects, all samples would need to be multiplexed together and run on the same lane. This may require an initial MiSeq run for library balancing. Additional lanes can be run if more sequencing depth is needed.

5. If you are unable to process all your RNA samples together and need to process them in batches, make sure that replicates for each condition are in each batch so that the batch effects can be measured and removed bioinformatically.

https://bioinformatics.cancer.gov/content/rna-seq

A good review:

Conesa *et al. Genome Biology* (2016) 17:13
DOI 10.1186/s13059-016-0881-8

Genome Biology

REVIEW Open Access

A survey of best practices for RNA-seq data analysis

Ana Conesa[1,2*], Pedro Madrigal[3,4*], Sonia Tarazona[2,5], David Gomez-Cabrero[6,7,8,9], Alejandra Cervera[10], Andrew McPherson[11], Michał Wojciech Szcześniak[12], Daniel J. Gaffney[3], Laura L. Elo[13], Xuegong Zhang[14,15] and Ali Mortazavi[16,17*]

Generic roadmap for expt design & analysis

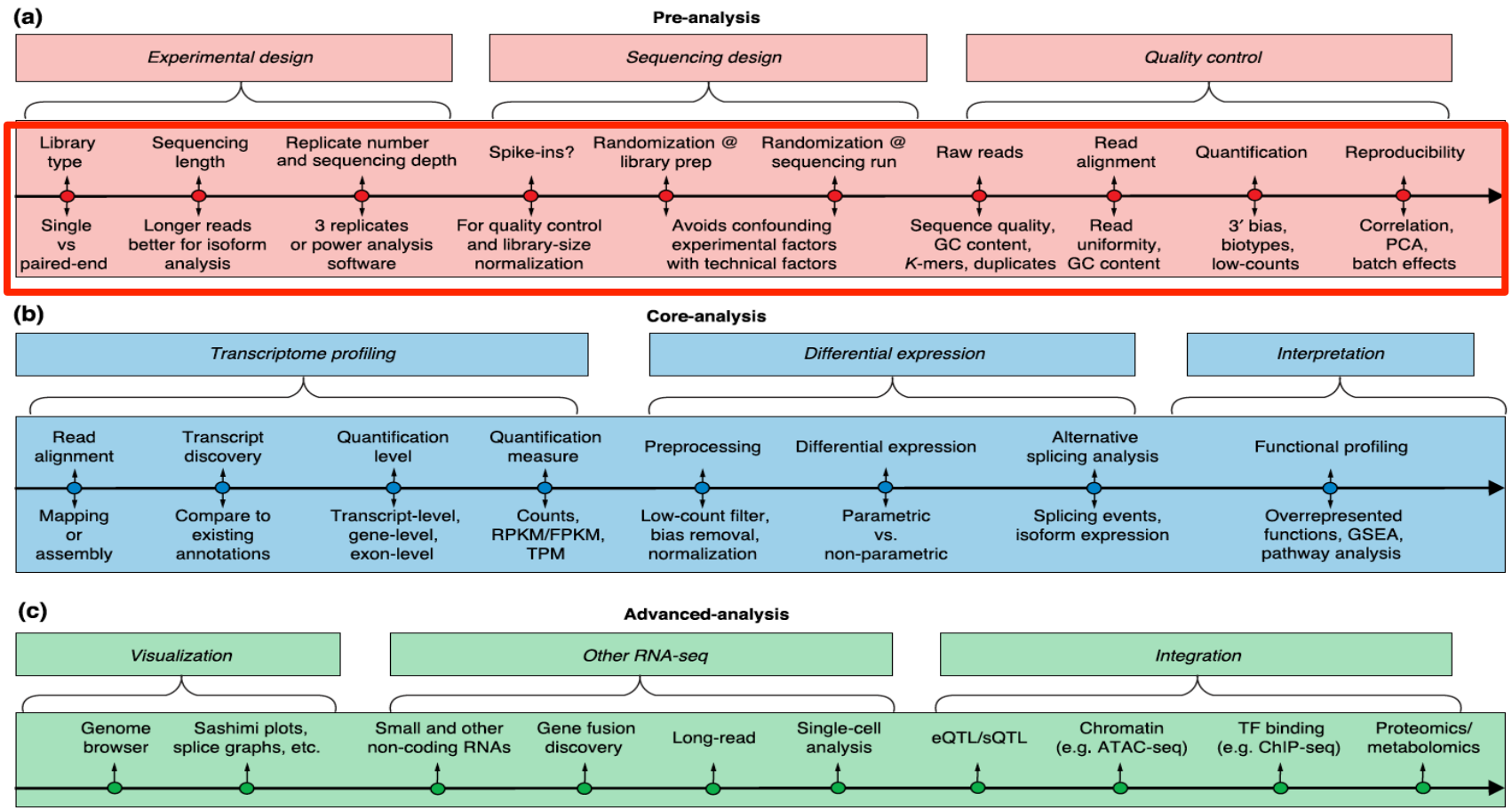


Fig. 1 A generic roadmap for RNA-seq computational analyses. The major analysis steps are listed above the lines for pre-analysis, core analysis and advanced analysis. The key analysis issues for each step that are listed below the lines are discussed in the text. **a** Preprocessing includes experimental design, sequencing design, and quality control steps. **b** Core analyses include transcriptome profiling, differential gene expression, and functional profiling. **c** Advanced analysis includes visualization, other RNA-seq technologies, and data integration. Abbreviations: *ChIP-seq* Chromatin immunoprecipitation sequencing, *eQTL* Expression quantitative loci, *FPKM* Fragments per kilobase of exon model per million mapped reads, *GSEA* Gene set enrichment analysis, *PCA* Principal component analysis, *RPKM* Reads per kilobase of exon model per million reads, *sQTL* Splicing quantitative trait loci, *TF* Transcription factor, *TPM* Transcripts per million

Samples vs Read depth

If on a tight budget, deciding between number of replicates vs sequencing depth, always higher replicates with lower sequencing depth leads to higher statistical power

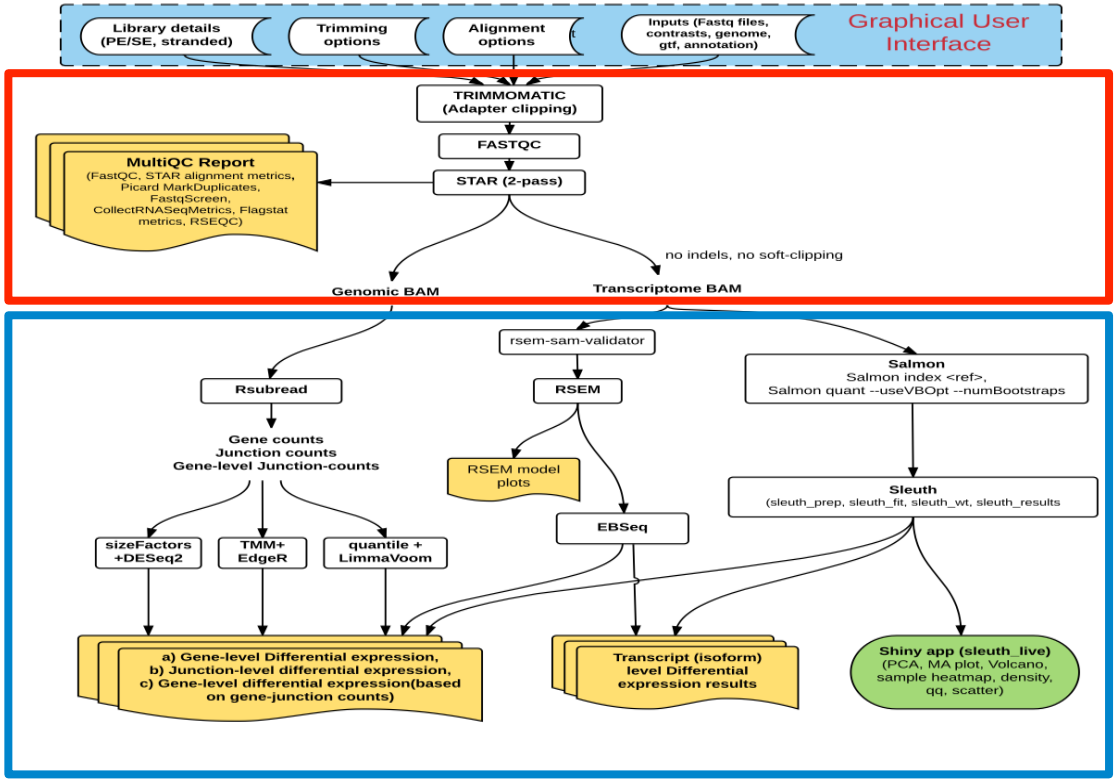
- 3M reads x 10 replicates = 30M reads yields 52% power
- 10mil reads x 3 replicates = 30M reads yields 33% power

Table 1 Statistical power to detect differential expression varies with effect size, sequencing depth and number of replicates

	Replicates per group		
	3	5	10
Effect size (fold change)			
1.25	17 %	25 %	44 %
1.5	43 %	64 %	91 %
2	87 %	98 %	100 %
Sequencing depth (millions of reads)			
3	19 %	29 %	52 %
10	33 %	51 %	80 %
15	38 %	57 %	85 %

Example of calculations for the probability of detecting differential expression in a single test at a significance level of 5 %, for a two-group comparison using a Negative Binomial model, as computed by the RNASeqPower package of Hart et al. [190]. For a fixed within-group variance (package default value), the statistical power increases with the difference between the two groups (effect size), the sequencing depth, and the number of replicates per group. This table shows the statistical power for a gene with 70 aligned reads, which was the median coverage for a protein-coding gene for one whole-blood RNA-seq sample with 30 million aligned reads from the GTEx Project [214]

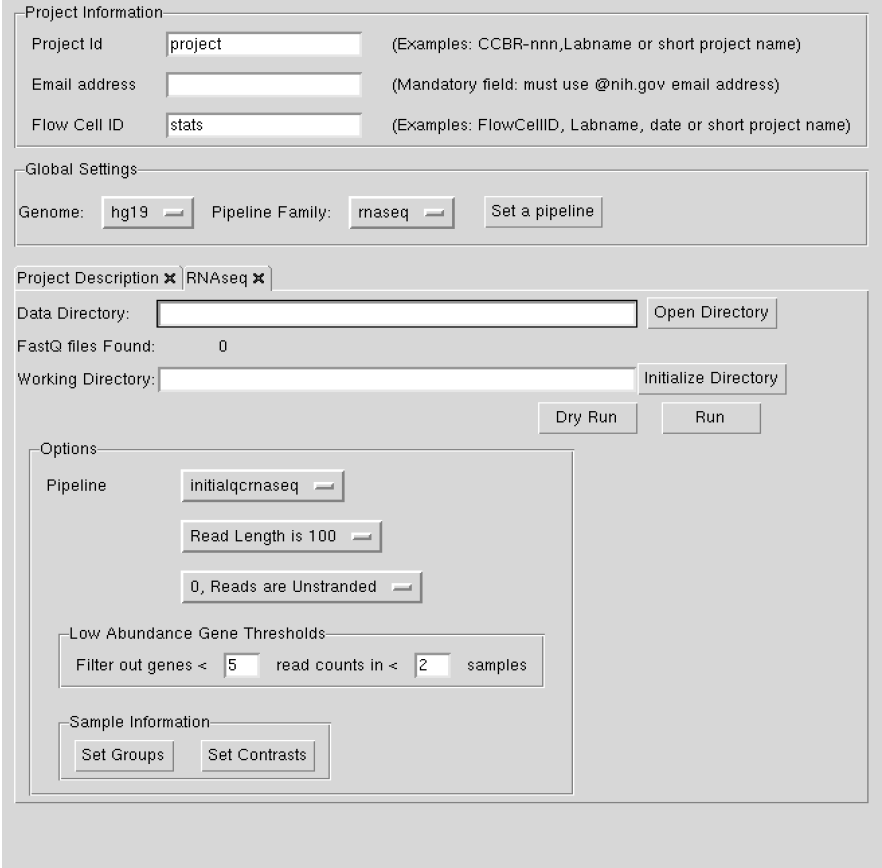
RNA-Seq Pipeline Workflow



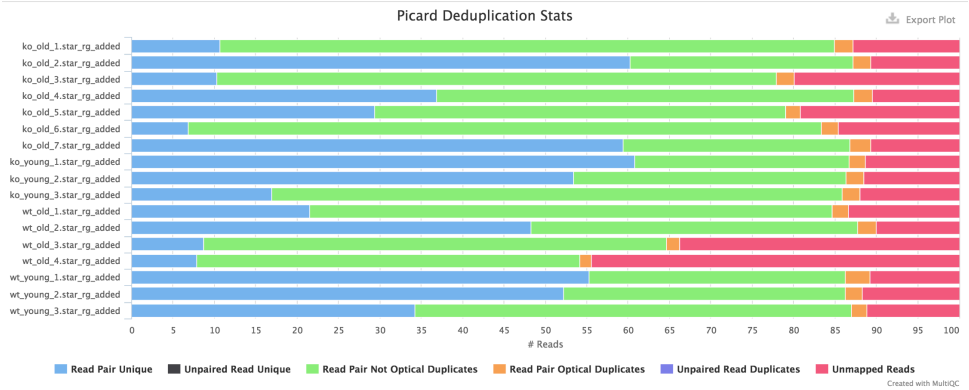
STEP1: INITIAL QC

STEP2: COUNTING & DEG

CCBR Pipeliner
(QC Report, DEG Analysis)

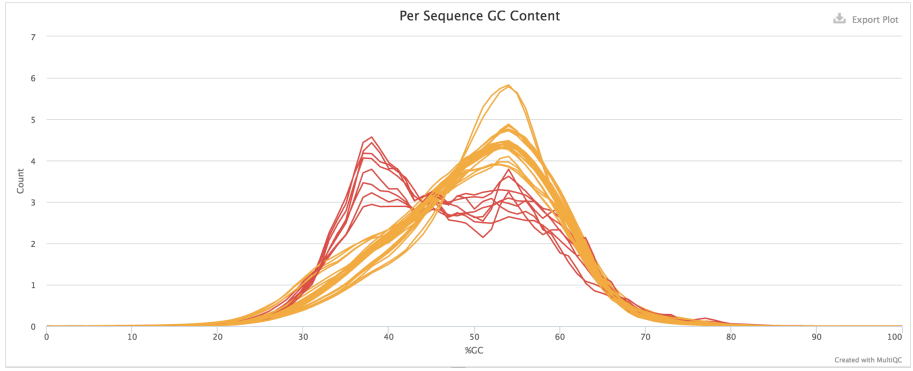


QC: Low RNA input
(0.1 – 1 ug total RNA or 10 - 100 ng isolated mRNA)



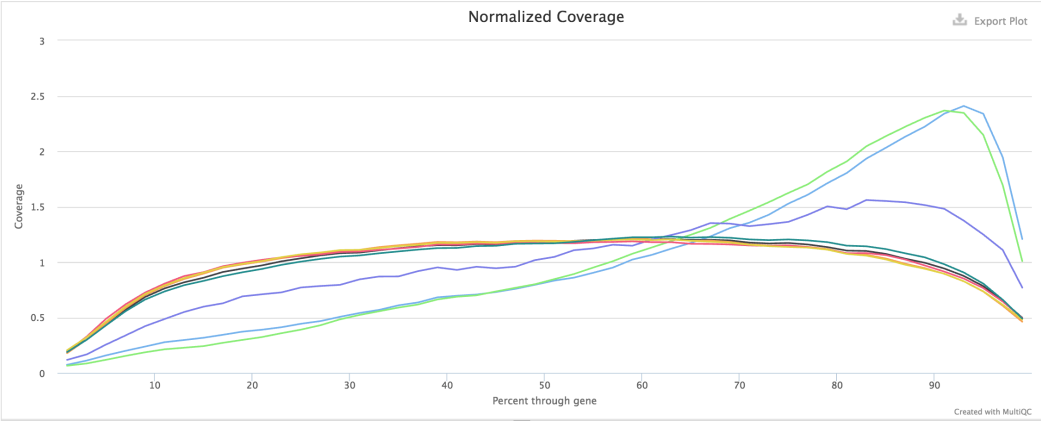
High duplication rates

GC Bias



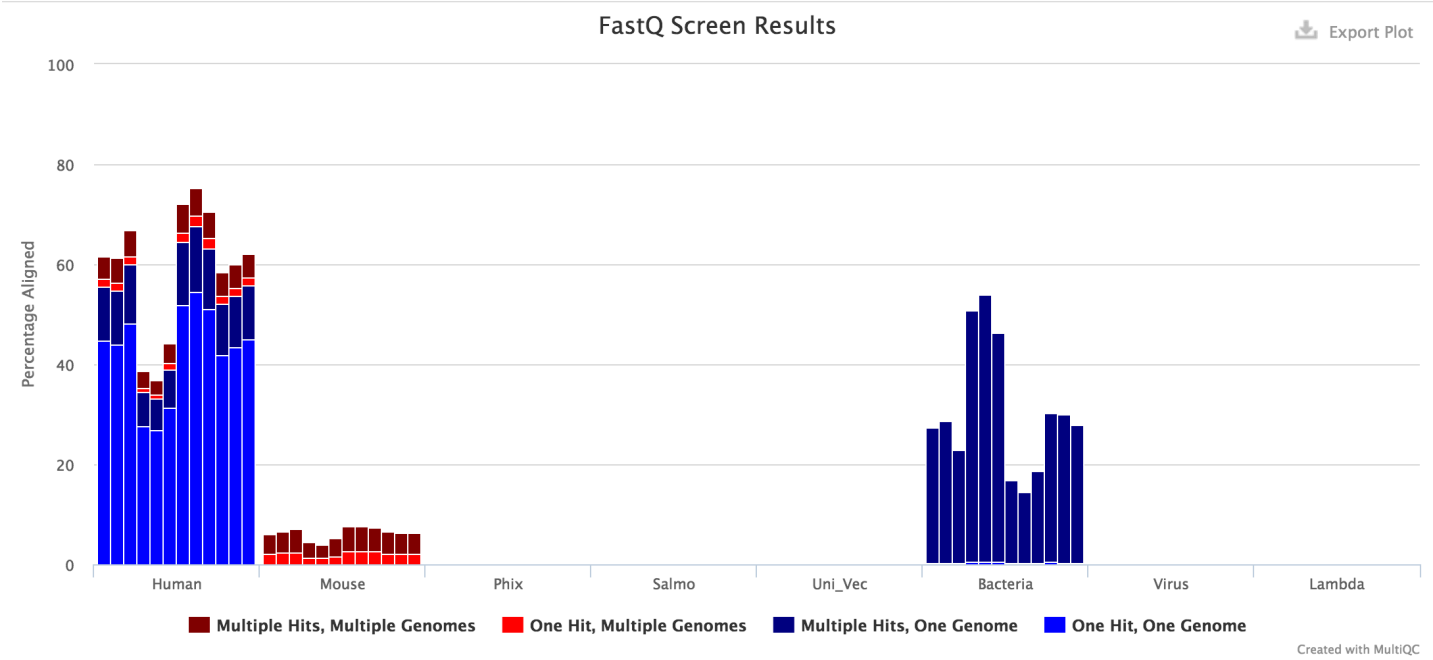
Clontech: 250pg – 10 ng RNA

QC: Poor RNA Quality
(RIN > 7, for FFPE or degraded, use ribominus)

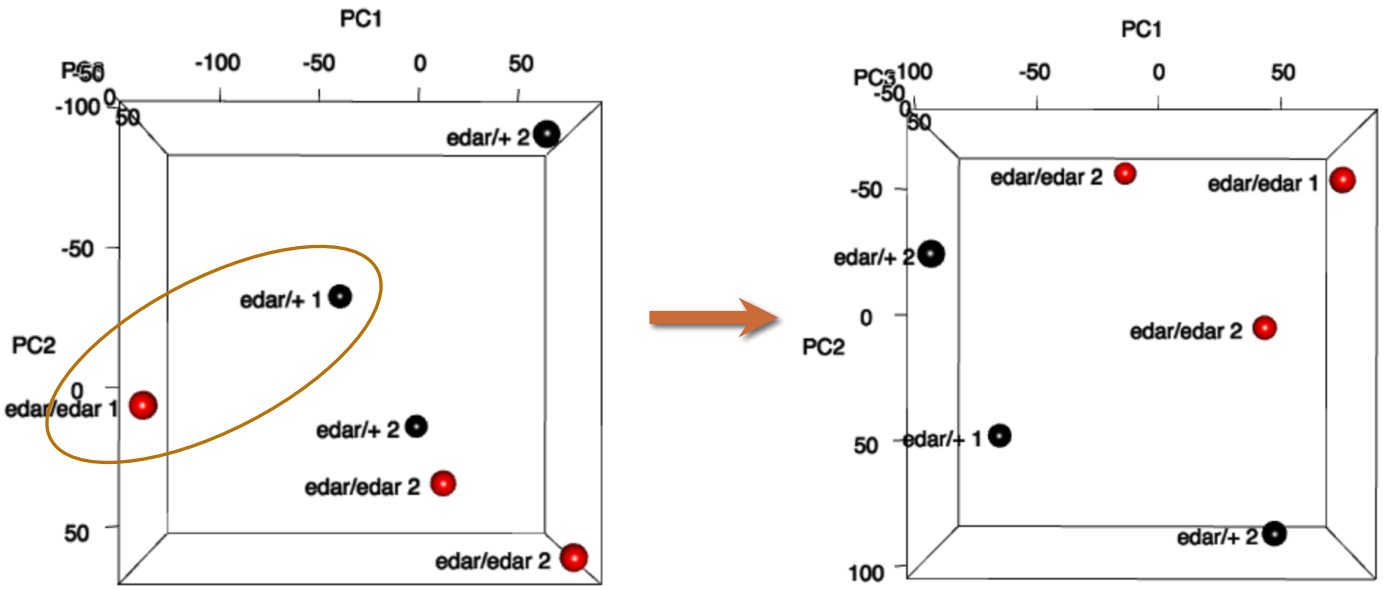


Degraded RNA showing 3’ bias in coverage

QC: Contamination



QC: Batch Effects



Litter effect: used batch removal

Take Home Message:

While you are planning your RNA-Seq experiment (not after), please come talk to us.

CCBR@mail.nih.gov