Low level Processing and Visualization of Genome Sequencing Data

GSND 5340Q, BMDA

W. Evan Johnson, Ph.D.
Professor, Division of Infectious Disease
Director, Center for Data Science
Rutgers University – New Jersey Medical School

2025-04-22

Motivating Example: X-linked Disease

Rare X-linked Disease



Rare X-linked Disease

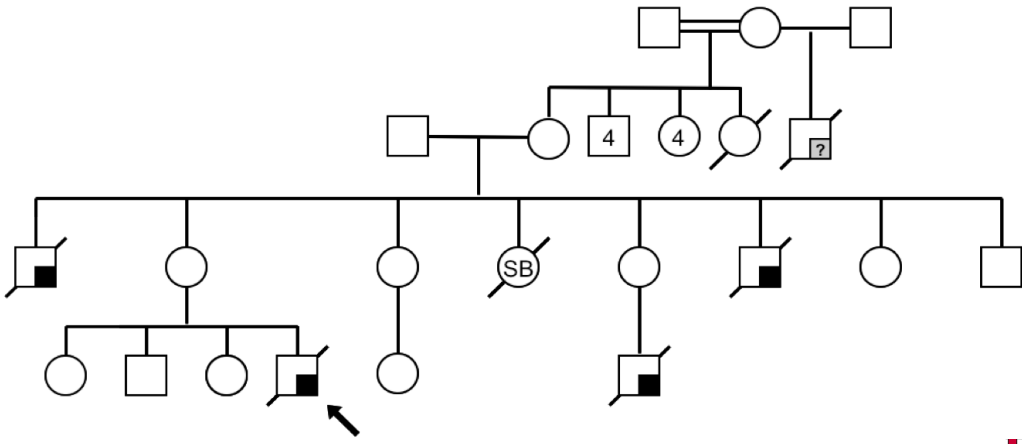
Uncle #1

Uncle #2

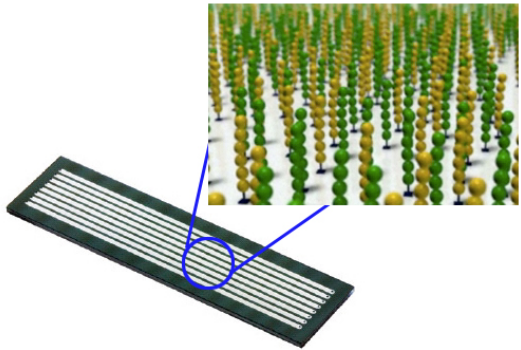
cousin

proband

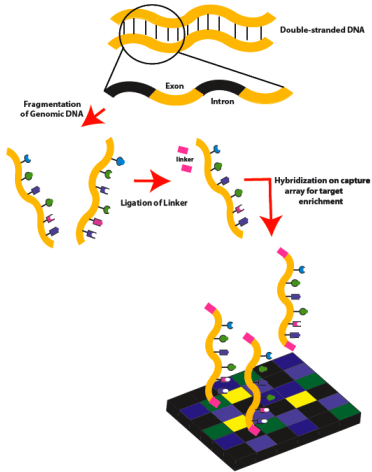
Rare X-linked Disease



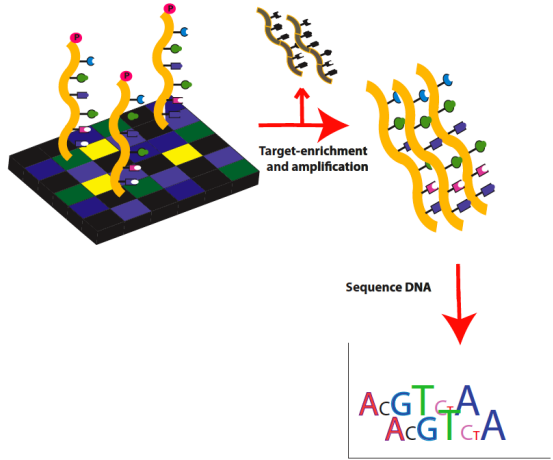
Exon Capture Sequencing



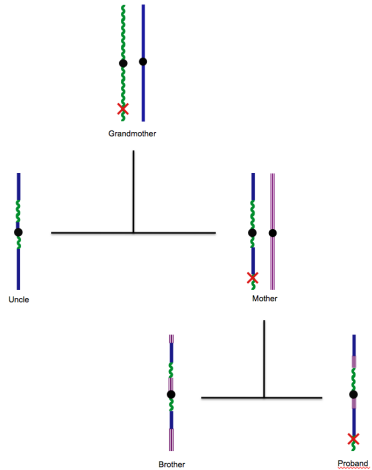
Exome Capture



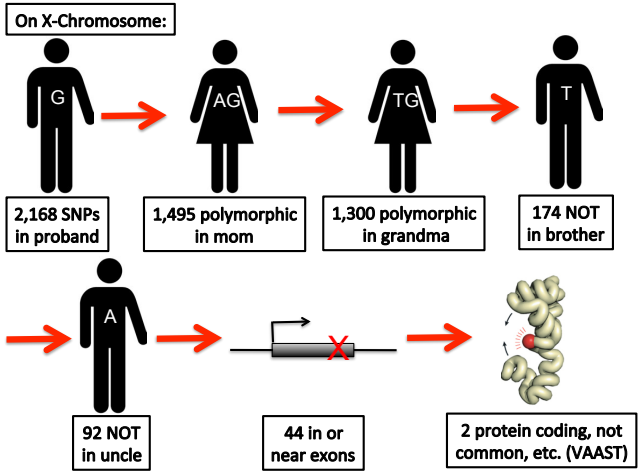
Exome Capture



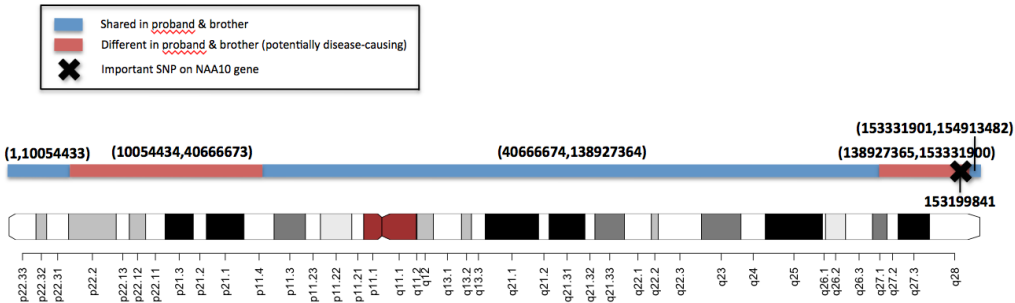
Rare X-linked Disease



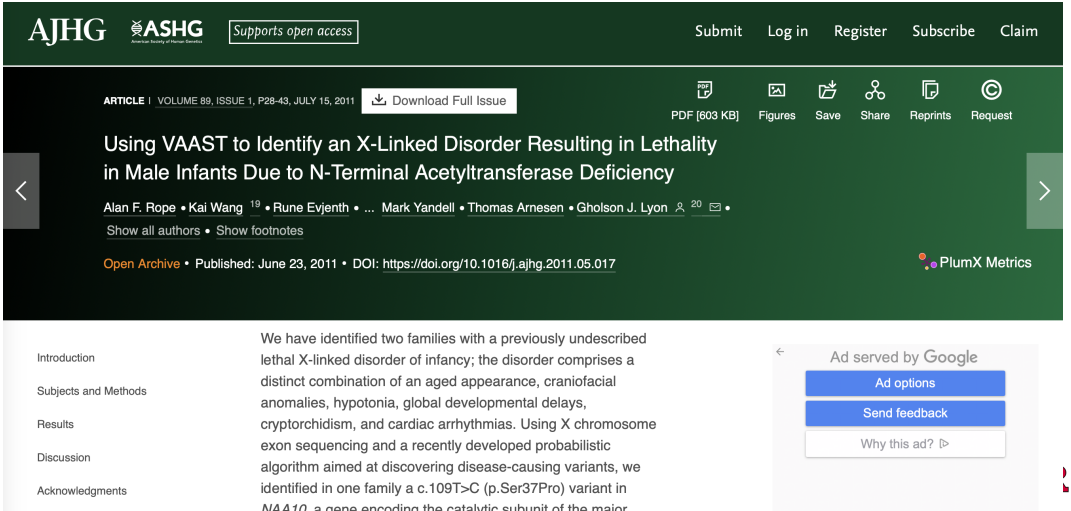
Rare X-linked Disease



Rare X-linked Disease



Rare X-linked Disease



AJHG ASHG Supports open access

Submit Log in Register Subscribe Claim

ARTICLE | VOLUME 89, ISSUE 1, P28-43, JULY 15, 2011 Download Full Issue

PDF [603 KB] Figures Save Share Reprints Request

Using VAAST to Identify an X-Linked Disorder Resulting in Lethality in Male Infants Due to N-Terminal Acetyltransferase Deficiency

Alan F. Rope • Kai Wang [19] • Rune Evjenth • ... Mark Yandell • Thomas Arnesen • Gholson J. Lyon [20] •

Show all authors • Show footnotes

Open Archive • Published: June 23, 2011 • DOI: https://doi.org/10.1016/j.ajhg.2011.05.017

PlumX Metrics

Introduction

Subjects and Methods

Results

Discussion

Acknowledgments

We have identified two families with a previously undescribed lethal X-linked disorder of infancy; the disorder comprises a distinct combination of an aged appearance, craniofacial anomalies, hypotonia, global developmental delays, cryptorchidism, and cardiac arrhythmias. Using X chromosome exon sequencing and a recently developed probabilistic algorithm aimed at discovering disease-causing variants, we identified in one family a c.109T>C (p.Ser37Pro) variant in *NAA10*, a gene encoding the catalytic subunit of the major

N-terminal acetyltransferase (NAA10)

N-terminal acetyltransferase (NAT):

- Common modification (~80-90% of human proteins)
- Depletion from cancer cells linked to cell cycle arrest and apoptosis (Starheim, *BMC Proc* 2009)
- NAT genes directly implicated as cause of genetic disease
- Mutation demonstrated a significantly impaired biochemical activity *in vitro*
- NAA10 lethal if knocked out of Drosophila

Generating Sequencing Data

Next-Generation Sequencing

- Expensive to purchase (hundreds of thousands $USD)
- Expensive to operate (e.g. reagents, flow cells)
- You can sequence your genome at 30X depth for <$1000 USD.

Roche 454

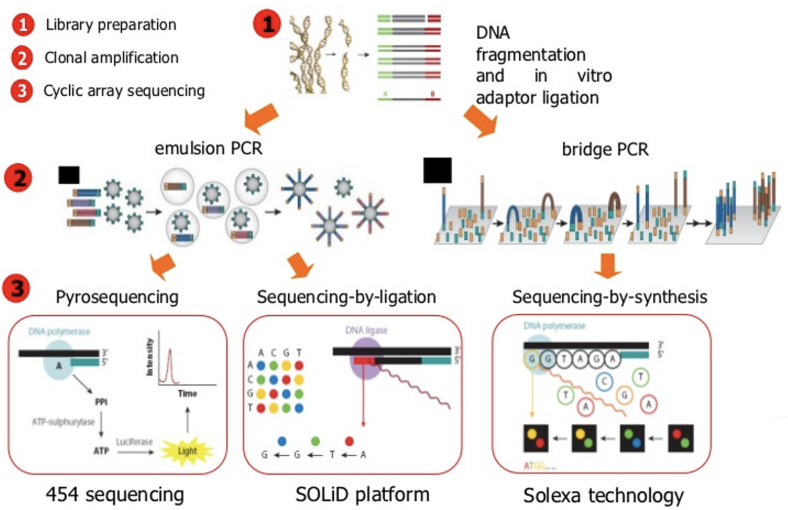
Ion Torrent

Illumina HiSeq 2500

Illumina NovaSeq 6000

Center for Data Science

Next-Generation Sequencing



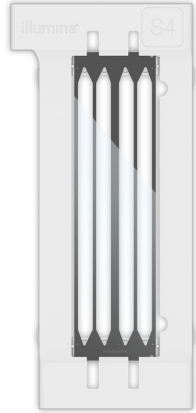
Illumina Sequencing

- Most common sequencing technology today
- Sequences any DNA
- Sequencing by synthesis method
- Sequences (reads) are short (<300bp)
- 2 gigabases - 6 terabases per run
- Hours to days to complete one run

For more information, you can watch the following:
https://www.youtube.com/watch?v=fCd6B5HRaZ8

Illumina Sequencing

- Sequencing occurs on a **flow cell**
- Each flow cell has 1 to 8 **lanes**
- Number of reads for overall flow cell varies
- Length of reads is fixed (e.g. 250 bp)
- Read format:
  - **Single end** - one read per molecule
  - **Paired end** - two reads per molecule
- **Multiplexing**: sequence many samples at once using molecular barcodes



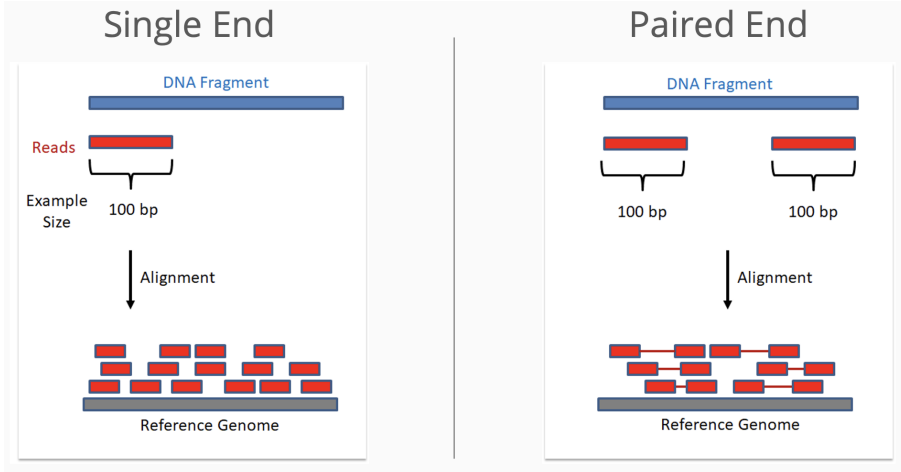
NovaSeq Flowcell
Courtesy of Illumina, Inc.

Sequencing Library Generation Workflow

Sequencing Workflow:

- Extract RNA/DNA from sample
  - If RNA, reverse transcribe to cDNA
- Size select using gel cut or random shearing
- PCR amplify DNA if concentration is low
- Add sequencing adapters
  - If multiplexing, use barcoded adapters
- Pool samples, load across flow lanes for sequencing
- Typically only perform 1, sequencing cores

Design Choice: Single End vs Paired End



Design Choice: Number of Reads

Table 1: Coverage and Read Recommendations by Application

Category	Detection or Application	Recommended Coverage (x) or Reads (millions)	References
Whole genome sequencing	Homozygous SNVs	15x	Bentley et al., 2008
	Heterozygous SNVs	33x	Bentley et al., 2008
	INDELs	60x	Feng et al., 2014
	Genotype calls	35x	Ajay et al., 2011
	CNV	1-8x	Xie et al., 2009; Medvedev at al., 2010
Whole exome sequencing	Homozygous SNVs	100x (3x local depth)	Clark et al., 2011; Meynert et al., 2013
	Heterozygous SNVs	100x (13x local depth)	Clark et al., 2011; Meynert et al., 2013
	INDELs	not recommended	Feng et al., 2014
Transcriptome Sequencing	Differential expression profiling	10-25M	Liu Y. et al., 2014; ENCODE 2011 RNA-Seq
	Alternative splicing	50-100M	Liu Y. et al., 2013; ENCODE 2011 RNA-Seq
	Allele specific expression	50-100M	Liu Y. et al., 2013; ENCODE 2011 RNA-Seq
	De novo assembly	>100M	Liu Y. et al., 2013; ENCODE 2011 RNA-Seq
DNA Target-Based Sequencing	ChIP-Seq	10-14M (sharp peaks); 20-40M (broad marks)	Rozowsky et al., 2009; ENCODE 2011 Genome; Landt et al., 2012

https://genohub.com/recommended-sequencing-coverage-by-application/

Design Choice: Sequencing Depth

Whole Exome

- Less expensive
- Nearly complete ascertainment of variation in the coding ~1% of the genome (i.e. exons)
- Will miss functional variants outside of the coding region

Design Choice: Sequencing Depth

Whole Exome Low Coverage Whole Genome

- Less expensive
- Reasonably good ascertainment of shared variation, but not unique variation

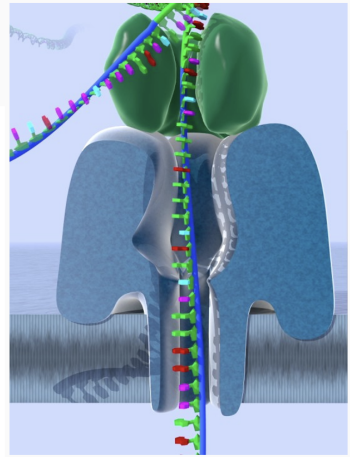
Design Choice: Sequencing Depth

Whole Exome Deep Whole Genome

- More expensive
- Capture most of the genetic information
- Sequence the entire genome of each subject

3rd Gen Sequencing: Oxford Nanopore

- "Real time single molecule"



https://www.youtube.com/watch?v=GUb1TZvMWsw

Sequencing Data Formats

Biological Data Formats are standardized

- Many different types of data
- Standard formats exist for many of them
- Always use/extend standard formats
- Don't save data in non standard formats when standards are available - No .xslx!
- For complete list of formats see: https://genome.ucsc.edu/FAQ/FAQformat.html

Sequencing Data Formats

format	data	tool(s)
FASTA	sequence of nucleotides	Samtools, Biopython
FASTQ	sequenced reads	FASTQC, Biopython
SAM/BAM/CRAM	aligned reads	Samtools, Deeptools, PySam
VCF	variant calls	vcftools bedtools
BED / BED-PE	genomic regions	bedtools
GFF	general features	Biopython, Manual Parsing
GTF	gene features	Biopython, Manual Parsing

Sequencing Data Formats

format	data	tool(s)
FASTA	sequence of nucleotides	samtools faidx
FASTQ	sequenced reads	-
SAM/BAM/CRAM	aligned reads	samtools
VCF	variant calls	vcftools bedtools
BED / BED-PE		bedtools
GFF		-
GTF	gene featur	-

Contains sequences of nucleotides / AA / bases

Sequencing Data Formats

format	data	
FASTA	s	aidx
FASTQ	sequ	
SAM/BAM/CRAM	aligned reads	samtools
VCF	variant calls	vcftools bedtools
BED / BED-PE	genomic regions	bedtools
GFF	general features	-
GTF	gene features	-

Contains positions: chromosome, start and end

Data Formats Example

EXAMPLE

Align reads to the reference, sort and index, call SNPs, extract the SNPs on chromosome Y overlapping with all the Alu elements.

Data Formats Example

EXAMPLE

Align reads to the reference, sort and index, call SNPs on chromosome Y overlapping with all the Alu elements.

FASTQ format

Data Formats Example

EXAMPLE

Align reads to the reference, sort and index, call SNPs on chromosome Y overlapping with all the Alu elements.

FASTA format

Data Formats Example

EXAMPLE

Align reads to the reference, sort and index, call SNPs, extract the SNPs on chromosor with all the Alu elements.

save in BAM format

Data Formats Example

EXAMPLE

Align reads to the reference, sort and index, call SNPs, extract the SNPs on chromosom VCF format ith all the Alu elements.

Data Formats Example

EXAMPLE

Align reads to the reference, sort and index, call SNPs, extract the SNPs on chromosome Y overlapping with all the Alu elements.

Read from GTF/GFF/BED

Data Formats Example

EXAMPLE

Align reads to the reference, sort and index, call SNPs, extract the SNPs on chromosome Y overlapping with all the Alu elements.

BWA/BOWTIE2

Data Formats Example

EXAMPLE

Align reads to the reference, sort and index, call SNPs, extract the SNPs on chromos g with all the Alu elements.

samtools

Data Formats Example

EXAMPLE

Align reads to the reference, sort and index, call SNPs, extract the SNPs on chromosome Y ove ne Alu elements.

samtools / GATK

Data Formats Example

EXAMPLE

Align reads to the reference, sort and index, call SNPs, extract the SNPs on chromosome Y overlapping with all the Alu elements.

samtools view

Data Formats Example

EXAMPLE

Align reads to the reference, sort and index, call SNPs, extract the SNPs on chromosome Y overlapping with all the Alu elements.

bedtools intersect

WEJ LAB

Raw Sequencing Data and QC

Center for Data Science

History and Evolution of Illumina Data Output

Illumina sequencers have given output in many different formats:

- Illumina .PRB and .INT files
  - Better access to raw data.
  - Base calling algorithms (Bravo and Irizarry, *Biometrics*, 2010)
  - Mapping algorithms (GNUMAP, NOVO)
  - Confusing formats; Large data files
- Illumina .FASTQ files
- Sanger .FASTQ files

Illumina .INT and .PRB

```
1   1   125 771 1651.8 2189.6 228.1 549.9   219.0 202.5  48.4 3016.8    127.8   6.1 2046.0 1709.3    155.4 215.1 1936.2 1472.2
1   1   478 16   1050.0 969.9 149.5 311.7     0.0   0.0  39.3  134.1     0.0   0.0   0.0   0.0      0.0   0.0   0.0   0.0    470.5   0.0
1   1   780 553   639.6 980.8 555.2 6412.8   1040.1 4408.7 750.3 638.1   946.2 4351.1 712.6 1244.1  2808.9 4518.5 329.0 946.2
1   1   123 685 -116.5 341.5 -14.0 -985.9    231.5 1090.0  88.3 -102.2   240.2 513.6   3.1 -282.3     19.1 -86.6  41.2  -6.5
1   1   61   934  40.7  87.3  38.5  21.3   16.4  31.7  100.9  68.8       41.4  29.4  40.4  30.1   79.1   3.5  52.3  75.9   53.0  43.6
1   1   866 972 2820.5 4698.9 435.8 8502.2 4740.3 4890.2 1491.5 1241.7 2137.5 2505.6 6653.8 4579.8 2243.2 3102.6 5157.2 2819.4
```

```
 40 -40 -40 -40  -40 -40 -40  40  -40 -40  40 -40  -40 -40  40 -40  -40 -40 -40  40  -40 -40 -40  40
 28 -28 -40 -40   -5  -5  -5  -5    -5  -5  -5  -5    -5  -5  -5  -5    -5  -5  -5  -5    -5  -5  -5  -5
-17 -16 -30  13  -40  26 -26 -40  -21  17 -20 -27   21 -21 -40 -40  -40 -40  40 -40 -27   24 -40 -26
-40  40 -40 -40  -40  40 -40 -40   -1   1 -40 -40   -0 -40  -0 -14   26 -26 -40 -40   40 -40 -40 -40
 -1 -40  -0 -10  -20 -18  16 -35   -1  -8  -2 -13   12 -34 -13 -26    5 -10 -12 -11   -4 -40  -1  -2
-27 -40 -40  27   40 -40 -40 -40  -40 -40  40 -40  -40 -40  40 -40  -40 -40  40 -40  -40 -40  40 -40
```

.fasta or .fa

```
>chrX
ttgaactcctgacctcaggtgatccgcccgccttgacctcccaaagcgct
cctgcctcagcctcccgagtagctgggactacaggtgcctgccaccatgc
....
caggctaatttttgtatttttagtagagacggggtttcaccatgttagc
caggatggtctcaatctcctgacctcatgatccgcctgcctcggcctccc
>chrY
tgtacactttaaatgggtgaattttatggaatgtgaattataCGTGTTGG
CTTGTAAAAAAAAATGATGGAGATGGAGACGTGACTCTAGCGTGAAGGGG
...
GTGGGGAGAGTAGATCTAGAGTGGAGACACCACTTTTAGGAGGTATGATC
cctgccaccatgcctggctaatttttgtatttttagtagagacagggttt
```

.fastq or .fq (or .fq.gz)

```
@HWI-EAS240_0001:2:1:1142:17571#0/1
CTCTCTTTTCTCCCCANGTCTCCTCATGACCATATCCNTGTTGTCCATTGTGTANGGNNNCTTGCGCTGCAACATC
+HWI-EAS240_0001:2:1:1142:17571#0/1
bababb`abbbbabbYB^[^^`_`_bbbbbbbbbb`_B`_``^`^bbbba^`^^BOOBBBNOOOObY^^bbbbbbb
@HWI-EAS240_0001:2:1:1142:6453#0/1
CAGGACGTGCACTATGNCATCCACGATGCAAGTCTTANCATTATTCAGGATACANATANNGGCAAACGTGTAATTA
+HWI-EAS240_0001:2:1:1142:6453#0/1
_U][aa_aa^^```^[B][][]]]S```Y``a`aaZ]B]]^^^[^```[OVY]XBNNNBB]]]]]L^^^^```[V`
@HWI-EAS240_0001:2:1:1142:19443#0/1
TCAGAAAACAGAAAGGTCATTTTCTTACTTCTTTGCANGATGCCACCCTCCAGANCAGNNAATTGCTACTTTGCAC
+HWI-EAS240_0001:2:1:1142:19443#0/1
bbbabb^^bbb^bbabb]bb]^]`]]]bbbbbbbbYYBYXOOOOObb`bbVY^HBOOOBBOJOLO_bbbb]b_b`a
```

.FASTQ Paired End

fastq_SRR1997469_1.fastq

```
@SRR1997469.1 1 length=36
CAGTCTTCTTAGAAATATCCACTTCGGAATAAAAGA
+SRR1997469.1 1 length=36
BBBBBFFFFF<FFFFFFFFFFFFFBFFFFFFFFFF
@SRR1997469.2 2 length=36
ACAGTTGAACGATCCTTTACAGANAGNAGNCTNGTA
+SRR1997469.2 2 length=36
<BBBBFFFBBF<BFF/<FFFFFF##############
...
```

fastq_SRR1997469_2.fastq

```
@SRR1997469.1 1 length=36
AGATAAGATGGTAATCTTTGATGGAGAACATTAAGA
+SRR1997469.1 1 length=36
BBBBBFFFFFFFFFFFFFFFFFFFFFFFFFFFFFF
@SRR1997469.2 2 length=36
ACTGGGAANCCTTCTGTCTAGCCTTATATGAAAAAA
+SRR1997469.2 2 length=36
BBB/BFFB#BB/FFFF/FFFFFFBBFFFFFFFFFBF
...
```

.FASTQ Comparison

```
 SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS.....................................................
 ..........................XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX.....................
 ............................IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII.....................
 ................................JJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJ.....................
 LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL.....................................................
 !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
  |                         |    |        |                              |                     |
33                        59   64       73                            104                   126

S - Sanger        Phred+33,  raw reads typically (0, 40)
X - Solexa        Solexa+64, raw reads typically (-5, 40)
I - Illumina 1.3+ Phred+64,  raw reads typically (0, 40)
J - Illumina 1.5+ Phred+64,  raw reads typically (3, 40)
   with 0=unused, 1=unused, 2=Read Segment Quality Control Indicator (bold)
   (Note: See discussion above).
L - Illumina 1.8+ Phred+33,  raw reads typically (0, 41)
```

https://en.wikipedia.org/wiki/Phred_quality_score

Sequence Quality Score (PHRED)

Phred quality scores are logarithmically linked to error probabilities

Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%
60	1 in 1,000,000	99.9999%

https://en.wikipedia.org/wiki/Phred_quality_score

Sequence Quality Score (PHRED)

Quality scores (Phred)

- Sanger Phred: Range=(0,40), $P = 1 - 10^{-(ASCII-33)/10}$
- Solexa: Range= (-5,40), $P = \frac{10^{(ASCII-64)/10)}}{1+10^{(ASCII-64)/10}}$
- Illumina 1.3: (0,40), $P = \frac{10^{(ASCII-64)/10)}}{1+10^{(ASCII-64)/10}}$
- Illumina 1.5: Range=(2,40), $P = 1 - 10^{-(ASCII-64)/10}$
- Illumina 1.8: Same as Sanger except Range=(0,41)

.FASTQ Comparison

> Sanger

	ASCII	Quality
!	33	0.0000
"	34	0.2057
#	35	0.3690
$	36	0.4988
%	37	0.6019
&	38	0.6838
'	39	0.7488
(	40	0.8005
)	41	0.8415
*	42	0.8741
+	43	0.9000
,	44	0.9206
-	45	0.9369

> Solexa

	ASCII	Quality
;	59	0.2403
<	60	0.2847
=	61	0.3339
>	62	0.3869
?	63	0.4427
@	64	0.5000
A	65	0.5573
B	66	0.6131
C	67	0.6661
D	68	0.7153
E	69	0.7597
F	70	0.7992
G	71	0.8337
H	72	0.8632
I	73	0.8882

> Illumina1.3

	ASCII	Quality
@	64	0.5000
A	65	0.5573
B	66	0.6131
C	67	0.6661
D	68	0.7153
E	69	0.7597
F	70	0.7992
G	71	0.8337
H	72	0.8632
I	73	0.8882
J	74	0.9091
K	75	0.9264
L	76	0.9406
M	77	0.9523
N	78	0.9617

> Illumina1.5

	ASCII	Quality
B	66	0.3690
C	67	0.4988
D	68	0.6019
E	69	0.6838
F	70	0.7488
G	71	0.8005
H	72	0.8415
I	73	0.8741
J	74	0.9000
K	75	0.9206
L	76	0.9369
M	77	0.9499
N	78	0.9602
O	79	0.9684
P	80	0.9749

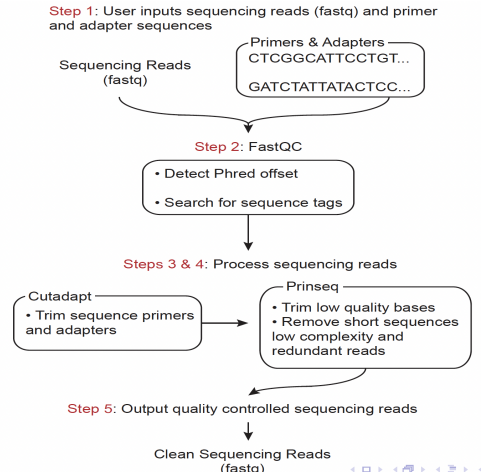
Quality Control

Need to preprocess the reads:

- Check for quality (FASTQC)
- Trim adapter and (Cutadapt, others)
- Remove duplicate reads, trim low complexity/quality bases/reads (Prinseq)
- Complete pipelines: NCBI Toolkit, QC-Chain, PathoQC (pathoscope.sourceforge.net), others

Note: Not comprehensive or updated!

Read Quality Checks

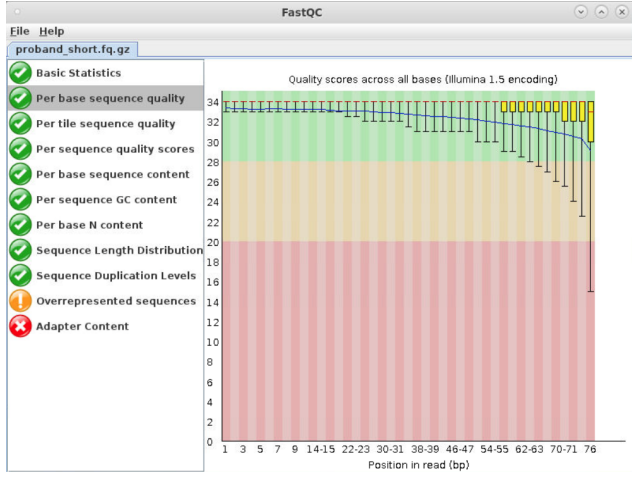


Read Quality Checks (Outdated!)

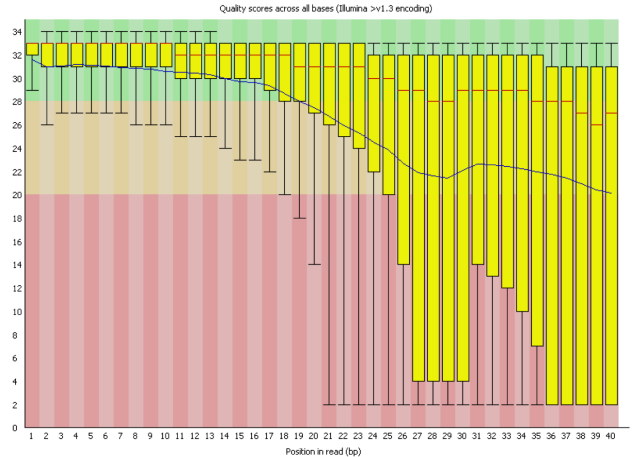
Comparison of some of the more popular QC pipelines:

QC Features	QCToolkit	QC-Chain	Cutadapt	Prinseq
Parallel computation	X	X		
Phred offset detection	X	X		
Tag sequence removal	X	X	X	
Poly-A/T tail trimming			X	X
PCR duplication filtering		X		X
Low complexity filtering				X
Homopolymer removal	X			X
GC content filtering		X		X
N/X content filtering				X

FASTQC


FastQC
File Help
proband_short.fq.gz
Basic Statistics
Per base sequence quality
Per tile sequence quality
Per sequence quality scores
Per base sequence content
Per sequence GC content
Per base N content
Sequence Length Distribution
Sequence Duplication Levels
Overrepresented sequences
Adapter Content
Quality scores across all bases (Illumina 1.5 encoding)
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
1 3 5 7 9 14-15 22-23 30-31 38-39 46-47 54-55 62-63 70-71 76
Position in read (bp)

FASTQC


Quality scores across all bases (Illumina >v1.3 encoding)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Position in read (bp)

FastQC Example

Interactive GUI from Amarel Desktop:

```
module spider fastqc
#module avail fastqc
module load FastQC
fastqc
```

FastQC Example

Running FastQC from command-line (single file):

```
fastqc myfastqfile.fq.gz --outdir=output/
```

Running FastQC from command-line (multiple files):

```
fastqc *.fq.gz --outdir=output/
```

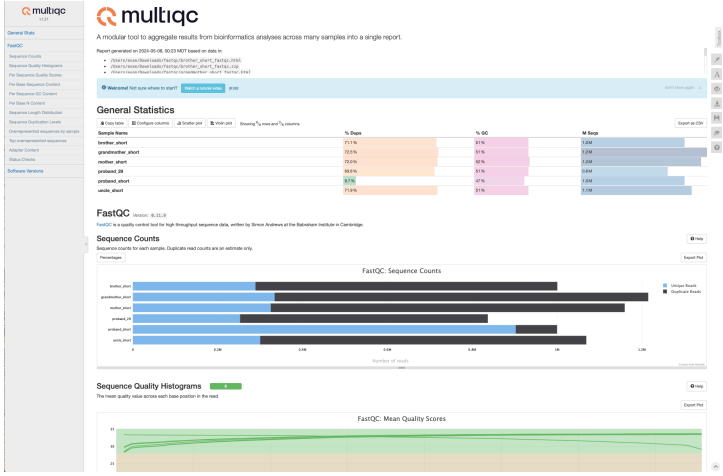
MultiQC Example

You can use `multiqc` to combine FastQC results.

```
pip install multiqc
cd my_fastqc_dir
multiqc .
```

MultiQC Example

MultiQC Example



Session info

```
sessionInfo()
```

```
## R version 4.4.2 (2024-10-31)
## Platform: aarch64-apple-darwin20
## Running under: macOS Sequoia 15.3.2
##
## Matrix products: default
## BLAS:   /Library/Frameworks/R.framework/Versions/4.4-arm64/Resources/lib/libRblas.0.dylib
## LAPACK: /Library/Frameworks/R.framework/Versions/4.4-arm64/Resources/lib/libRlapack.dylib;  LAPACK version 3.12.0
##
## locale:
## [1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8
##
## time zone: America/New_York
## tzcode source: internal
##
## attached base packages:
## [1] stats     graphics  grDevices utils     datasets  methods   base
##
## loaded via a namespace (and not attached):
##  [1] compiler_4.4.2    fastmap_1.2.0     cli_3.6.4         tools_4.4.2
##  [5] htmltools_0.5.8.1 rstudioapi_0.17.1 yaml_2.3.10       rmarkdown_2.29
##  [9] knitr_1.50        xfun_0.51         digest_0.6.37     rlang_1.1.5
## [13] evaluate_1.0.3
```