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Advanced Sequencing Technologies & Applications

<http://meetings.cshl.edu/courses.html>

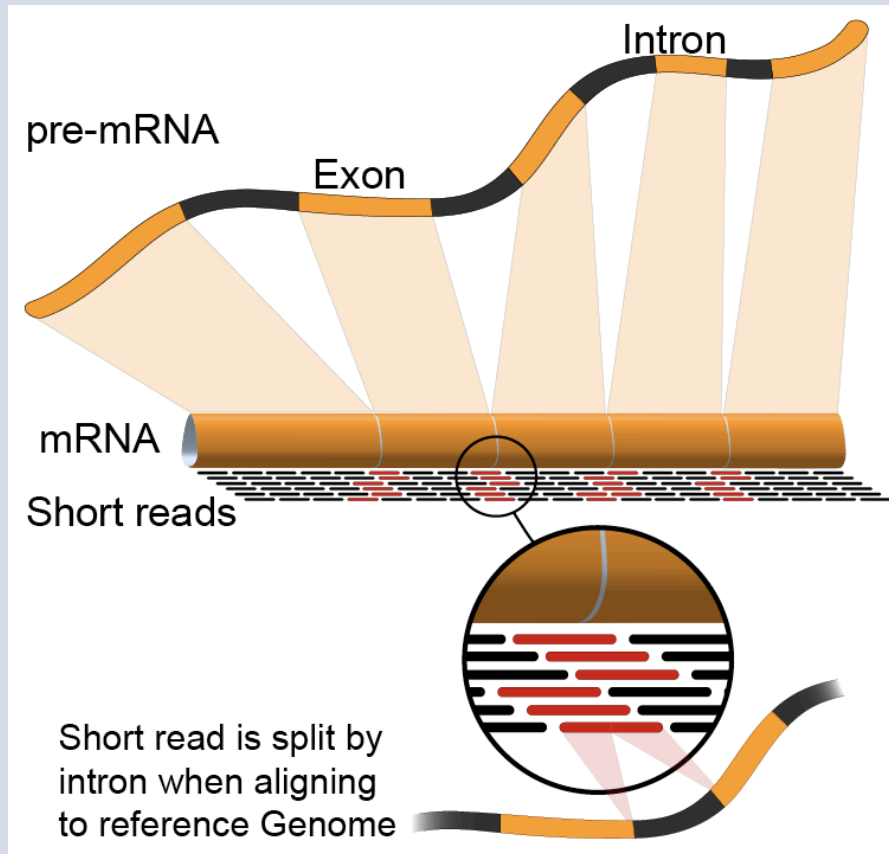


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RNA-Seq Module 1

Introduction to RNA sequencing (lecture)

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Advanced Sequencing Technologies & Applications
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Learning Objectives of Tutorial

- Install commonly used RNA-seq tools (Samtools, bam-readcount, HISAT2, StringTie, gffcompare, htseq-count, FastQC, picard-tools, Flexbar, R, Bioconductor, Ballgown, edgeR, ...)
- Obtain a reference genome
- Obtain gene/transcript annotations
 - Understand GTF file format
- Index reference genome files for use with aligners
- Obtain and explore raw sequence data
 - Understand fasta/fastq format

The most common problems encountered while working on the tutorials

- Type short commands carefully if you like, but in order to get through all the steps smoothly, it is safer to copy and paste from the tutorial files
- Copy/Paste errors
 - Learn the short cuts for copying/pasting on your system and use them (e.g. `<command><c>` & `<command><v>` on Mac)
 - Make sure you copy the entire command. Watch out for commands that span across multiple lines
- Being in the wrong directory at the wrong time
 - The simplest way to avoid this is only change directories as instructed
 - If you do change directories to look around, make sure you go back before continuing with commands
- Not having the `$RNA_HOME` environment variable set
 - Make sure you check this when logging in:
 - `echo $RNA_HOME`
 - If it is not defined do this:
 - `export RNA_HOME=~/.workspace/rnaseq`
 - Then add this to your `.bashrc` file so that you don't have to worry about it again

Introduction

- This presentation provides a brief description of tutorial steps
- The wiki contains more complete instructions
- Lines beginning with “#” are comments
- All other lines are commands that will be pasted and executed from a linux terminal or R tutorial
- Each command is annotated with comments except that basic familiarity with linux is assumed
 - e.g. You should know that ‘mkdir’ means to ‘make a directory,’ ‘cd’ means to ‘change directory’, etc.
- Some reference materials for linux can be found here:
 - <http://files.fosswire.com/2007/08/fwunixref.pdf>
 - <http://vic.gedris.org/Manual-ShellIntro/1.2/ShellIntro.pdf>
 - www.nettech.in/course/Basic%20Commands.pdf

1-i. Installation

- Installation instructions are provided for:
 - Samtools
 - <http://www.htslib.org/download/>
 - bam-readcount
 - <https://github.com/genome/bam-readcount>
 - HISAT2
 - <https://ccb.jhu.edu/software/hisat2/index.shtml>
 - StringTie
 - <https://ccb.jhu.edu/software/stringtie/>
 - Gffcompare
 - <http://ccb.jhu.edu/software/stringtie/gff.shtml>
 - htseq-count
 - <https://pypi.python.org/pypi/HTSeq>
 - FastQC
 - <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>
 - picard-tools
 - <https://github.com/broadinstitute/picard>
 - Flexbar
 - <https://github.com/seqan/flexbar>
 - R
 - <https://www.r-project.org/>
 - Bioconductor
 - <https://www.bioconductor.org/install/>
 - Ballgown (Bioconductor package)
 - <http://bioconductor.org/packages/release/bioc/html/ballgown.html>
 - edgeR (Bioconductor package)
 - <https://bioconductor.org/packages/release/bioc/html/edgeR.html>

1-ii. Obtain reference genome

- All reference files are obtained from Ensembl
 - ftp://ftp.ensembl.org/pub/release-86/fasta/homo_sapiens/dna/
 - This step downloads reference human genome files from Ensembl
 - The GRCh38 build of the human genome is used
 - This is the latest version of the human reference
- For the tutorial, a single chromosome is used (chr. 22)
 - The reason for this is to reduce run time for the tutorial
 - Instructions for downloading all chromosomes are provided

1-iii. Obtain known transcript annotations

- All annotation files are obtained from Ensembl
 - <http://useast.ensembl.org/info/data/ftp/index.html>
 - There are many other ways to obtain gene annotation files. For example:
 - UCSC Genome Browser, Ensembl API, BioMart, Entrez, Galaxy, etc. could also be used
- You will download GTF files describing human transcripts (exon coordinates, gene ids, gene symbols, etc.)
- Descriptions of the GTF file format can be found here:
 - <http://genome.ucsc.edu/FAQ/FAQformat.html#format4>

1-iv. Create Indexed reference genome

- Before sequences can be mapped to the genome, it must be ‘indexed’ in a way that is compatible with the aligner being used
 - Since we are using HISAT2 for alignment, we will need an index built for that purpose
 - Other RNA-seq aligners will have their own indexing utility
 - E.g. TopHat and STAR.
 - Do not use an index created for another aligner

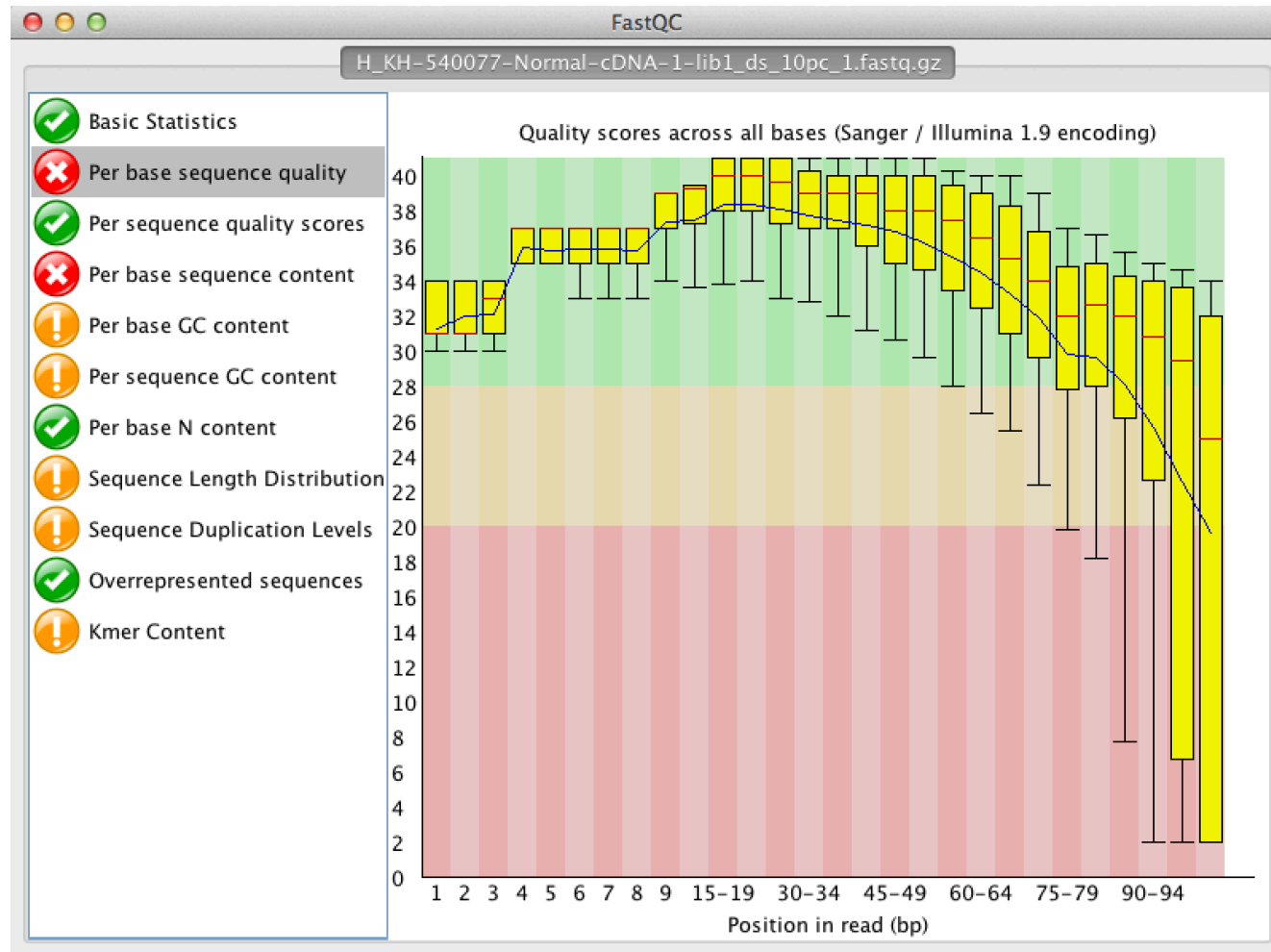
1-v. Obtain RNA-seq data

- For purposes of the tutorial, the test data has been pre-filtered
 - Identified reads that appear to match transcripts on a single chromosome
- The test data corresponds to two RNA sources
 - The Universal Human Reference (UHR) and Human Brain Reference (HBR)
 - Each sample also included one of two ERCC RNA “spike-in” mixes (Mix1 or Mix2)
 - Each RNA source was sequenced in triplicate to create six independent Illumina sequence libraries (**‘UHR_Rep1_Mix1’, ‘UHR_Rep2_Mix1’, ‘UHR_Rep3_Mix1’, ‘HBR_Rep1_Mix2’, ‘HBR_Rep2_Mix2’, and ‘HBR_Rep3_Mix2’**)
- The input data is provided in ‘fastq’ format:
 - http://en.wikipedia.org/wiki/FASTQ_format

1-v. Obtain RNA-seq data (cont'd)

- Universal Human Reference (UHR):
 - A pool of 10 human cell lines. This sample was purchased from Strategene (Agilent Technologies)
 - <http://www.genomics.agilent.com/en/References-Controls/Universal-Reference-RNAs/?cid=AG-PT-172&tabId=AG-PR-1217>
- Human Brain Reference (HBR):
 - A pool of brain tissue from multiple brain regions from multiple human donors. This sample was purchased from Ambion (Life Technologies).
 - <http://www.lifetechnologies.com/order/catalog/product/AM6050>
- External RNA Reference Consortium (ERCC):
 - ERCC reference RNA spike-ins purchased from Ambion (Life Technologies).
 - <http://www.lifetechnologies.com/order/catalog/product/4456739>
 - The UHR samples used ERCC Mix1. The HBR samples used ERCC Mix2.
- In this tutorial we will compare the three UHR libraries vs three HBR libraries (6 samples in total)

1-vi. Pre-Alignment QC with FastQC



Break