RNA-seq: experimental design

pre-mRNA

Exon

mRNA

Intron

Short reads

Short read is split by intron when aligning to reference Genome

http://upload.wikimedia.org/wikipedia/commons/0/01/RNA-Seq-alignment.png
Transcriptomics (RNA-Seq)

• The process of sequencing the “transcriptome”

• Uses include –
  ○ Differential Gene Expression
    Quantitative evaluation and comparison of transcript levels
  ○ Transcriptome assembly
    Building the profile of transcribed regions of the genome, a qualitative evaluation.
  ○ Can be used to help build better gene models, and verify them using the assembly
  ○ Metatranscriptomics or community transcriptome analysis
Outline

- Library preparation and sequencing with Illumina
- Experimental and Practical Considerations
RNA-Seq library prep

1. mRNA or total RNA
2. Remove contaminant DNA
3. Fragment RNA
4. Reverse transcribe into cDNA
5. Ligate sequence adaptors

http://rnaseq.uoregon.edu/#rna-prep

5. Ligate sequence adaptors

6. Select a range of sizes

http://rnaseq.uoregon.edu/#rna-prep

Illumina: Sequencing by Synthesis

Library Preparation

DNA (0.1-5.0 µg)

Cluster Growth

Single molecule array

Image Acquisition

Sequencing

Base Calling

5' T G C T A C G A T

3' T G C T A C G A T...

https://www.youtube.com/watch?v=fCd6B5HRaZ8&t=3s
Number of clusters \( \sim \) Number of reads
Number of sequencing cycles \( \sim \) Length of reads
Illumina: Sequencing Platforms

https://www.illumina.com/systems/sequencing-platforms.html
Other Sequencing Platforms

Oxford Nanopore (MinION): https://nanoporetech.com/
Pacific Biosciences: http://www.pacb.com/
Outline

- Library preparation and sequencing with Illumina
- Experimental and Practical Considerations
Experimental and Practical considerations

1. Experimental Design
2. Poly(A) enrichment or ribosomal RNA depletion?
3. Single-end or Paired-end data?
4. Stranded libraries?
5. How much sequencing data to collect?
6. Multiplexing
1. Experimental design

- **Technical replicates**: Illumina has low technical variation unlike microarrays, hence technical replicates are unnecessary.

- **Biological replicates**, are absolutely essential. Have at least 3!

- **Batch effects** are still a problem. Be consistent!

- For differential gene expression, **pooling** RNA from multiple biological replicates can be tricky; do so only if you have multiple pools from each experimental condition.
2. Poly(A) enrichment or ribosomal RNA depletion?

 Depends on which RNA entities you are interested in…

✦ For differential gene expression, it is best to enrich for Poly(A)+

• EXCEPTION – If you are aiming to obtain information about long non-coding RNAs, then do a ribosomal RNA depletion.
3. Single-end or Paired-end data?

Depends on your goals, paired-end reads are better for reads that map to multiple locations, for assemblies and for splice isoform differentiation.
Options for sequencing

- **SE** - Single end dataset => Only Read1
- **PE** - Paired-end dataset => Read1 + Read2
  - can be 2 separate FASTQ files or just one with interleaved pairs

http://tucf-genomics.tufts.edu/home/faq
✓ SE - Single end dataset => Only Read1
✓ PE - Paired-end dataset => Read1 + Read2
  • can be 2 separate FASTQ files or just one with interleaved pairs
✓ Fragment length: ~300-500bp
✓ Read length: 50bp - 250bp, depends on the sequencer (HiSeq2500, MiSeq, NextSeq)
3. Single-end or Paired-end data?

Depends on your goals, paired-end reads are better for reads that map to multiple locations, for assemblies, and for splice isoform differentiation.

- For differential gene expression, which one you pick depends on-
  - If you are specifically interested in isoform-level differences
  - The abundance of paralogous genes in your system of interest
  - Your budget, paired-end data is usually 2x more expensive
4. Stranded libraries?

Stranded libraries are now standard with Illumina’s TruSeq stranded RNA-Seq kits. This means that with a great amount of certainty you can identify which strand of DNA the RNA was transcribed from.

3 types of libraries –

- Reverse (firststrand) – reads resemble the complementary sequence (TruSeq)
- Unstranded
- Forward (secondstrand) – reads resemble the gene sequence
5. How much sequencing data to collect?

- Only ~2% of the human genome transcribes protein-coding RNA
- Some mRNAs will be much more abundant than others
- Some genes are much longer than others

Recommendations:

- For human samples ~30-50 million reads/sample (ENCODE guidelines)
- Modify that number based on the size of your transcriptome (crude estimate)
- If working with a tight budget:
  
  More replicates >> More reads (for standard differential expression analysis)
6. Multiplexing (with barcodes and indices)

- Charges for sequencing are usually per lane of the flow cell
- Each lane generates ~150 million reads
- For RNA-Seq, the required data per sample is much lower than that
- Sequencing of multiple samples per lane possible with addition of indices (within the Illumina adapter) or special barcodes (outside the Illumina adapter).
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